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APPLICATION FOR LETTERS PATENT

FOR

PRODRUGS ACTIVATED BY TARGETED CATALYTIC PROTEINS

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Prodrugs Activated By Targeted Catalytic Proteins

Related Applications

This application is a continuation-in-part of U.S. Application Serial No. 07/773,042, filed October 10, 1991, incorporated herein by reference. This application is also a continuation-in-part of U.S. Application Serial No. 740,501, filed August 5, 1991, hereby incorporated by reference. This application is also a continuation-in-part of U.S. Application Serial No. 190,271, filed May 4, 1988, a continuation-in-part of PCT/US89/01951, filed May 4, 1989, a continuation-in-part of U.S. Application Serial No. 700,210, filed June 12, 1991, a continuation-in-part of PCT/US89/01950, filed May 4, 1989, a continuation-in-part of U.S. Application Serial No. 07/761,868, filed November 4, 1991, and a continuation-in-part of U.S. Application Serial No. 498,225, filed March 23, 1990; and, each of these predessor applications is also incorporated herein by reference.

Field of the Invention

The present invention provides methods and compounds for providing suitable prodrugs of cytotoxic agents that are activated by enzymes or catalytic antibodies.

Background of the Invention

Many pharmaceutical compounds such as antiviral, immunosuppresive, and cytotoxic cancer chemotherapy agents generally have undesirable toxic effects on normal tissues. Such effects, which include damage to bone marrow (with consequent impairment of blood cell production) and gastrointestinal mucosa, alopecia, and nausea, limit the dose of pharmaceutical compound that can be safely administered and thereby reduce the potential efficacy.

Prodrugs Of Antineoplastic Agents

a. Nucleoside Analogs

A number of nucleoside analogs have utility as antitumor agents, including fluorouracil, fluorodeoxyuridine, fluorouridine, arabinosyl cytosine, mercaptopurine riboside, thioguanosine, arabinosyl fluorouracil, azauridine, azacytidine, fluorcytidine, fludarabine. Such drugs generally act by conversion to nucleotide analogs that either inhibit biosynthesis of important nucleotides or that are incorporated into nucleic acids, resulting in defective RNA or DNA.

5-Fluorouracil (5-FU) is a major antineoplastic drug with clinical activity in a variety of solid tumors, such as cancers of the colon and rectum, head and neck, liver, breast, and pancreas. 5-FU has a low therapeutic index. The size of the dose that is administered is limited by toxicity, reducing the potential efficacy that would be obtained if higher concentrations could be attained near tumor cells.

5-FU must be anabolized to the level of nucleotides (e.g., fluorouridine- or fluorodeoxyuridine-5'-phosphates) in order to exert its potential cytotoxicity. The nucleosides corresponding to these nucleotides (5-fluorouridine and 5-fluoro-2'-deoxyuridine) are also active antineoplastic agents, and in some model systems are substantially more potent than 5-FU, probably because they are more readily converted to nucleotides than is 5-FU.

AraC, also called arabinosylcytosine, 1-β-D-arabinofuranosylcytosine, cytarabine, cytosine-β-D-arabinofuranoside and β-cytosine arabinoside, is a widely used anti-cancer drug, albeit with some major disadvantages (see below). Currently AraC is used to treat both myelogenous and lymphocytic leukemias and non-Hodgkin's lymphomas. Used alone it has resulted in a 20-40% remission of acute leukemia and, in combination with other chemotherapeutic agents, has yielded greater than 50% remission (Calabresi, et al., "In The Pharmacological Basis of Therapeutics". Eds. Gilman, A.G., et al., New York: Macmillian Publishing Company, (1985):1272).

One of the disadvantages of AraC as a cancer drug is its rapid catabolism by deaminases. Human liver contains high levels of deoxycytidine deaminase which converts AraC to Ara-Uracil, an inactive metabolite. This rapid catabolism results in a $t_{1/2}$ in humans of 3-9 minutes following parenteral administration (Baguley, et al., Cancer Chemotherapy Reports 55 (1971):291-298). Compounding this problem, only cells undergoing DNA synthesis are susceptible to the drug's effect and therefore, one must maintain a toxic concentration until all cells of an asynchronously growing tumor pass through S-phase. Unfortunately, this means that the optimum dose schedule of AraC involves a slow intravenous infusion over many hours on each of 5 days, thus requiring a hospital stay. Prolonged application leads to the major problem of general toxicity among rapidly dividing normal cells, leading to bone marrow suppression, infection, and hemorrhage. Another problem encountered using this drug is the resistance to AraC eventually developed by cells, presumably due to selection of cells with low kinase activity, or an expanded pool of deoxy CTP.

Prodrug derivatives of AraC have been synthesized in order to: 1) protect AraC from rapid degradation by cytidine deaminase; 2) act as molecular depots of AraC and thereby simplify drug dose schedules; 3) act as carrier molecules for transport on serum proteins and facilitate cellular uptake; or 4) overcome resistance of cells with low kinase activity. AraC derivatives substituted at the 5' position of the arabinose or the N4 position of the cytidine ring have been found to be cytidine deaminase-resistant. Acting as carrier molecules that protect AraC from degradation by cytidine deaminase, lipophilic 5'-ester derivatives (Neil, et al., Biochem. Pharmacol. 21 (1971):465-475; Gish, et al., J. Med. Chem. 14 (1971):1159-1162) and N4-acyl derivatives (Aoshima, et al., Cancer Res. 36 (1976):2726-2732) of AraC have been shown to possess higher antitumor activity than AraC in leukemic mice.

All of the above prodrug derivatives are designed to be administered systemically as the parent drug itself is administered. The side effects of the prodrug arising out of the non-

tumor-specific toxicity are very similar, if not identical to the systemic application of the parent drug, Ara-C. These prodrugs are presumably acting as molecular depots of Ara-C and thus prolonging the time of drug availability.

Some prodrugs of other antineoplastic nucleoside analogs are also known. Such prodrugs are generally acyl derivatives of the nucleoside analogs; the acyl groups are removed by endogenous esterase activity following administration. Some of these prodrugs of arabinosyl cytosine (Neil, et al., Cancer Research 30 (1970):1047-1054; Neil, et al., Biochem Pharmacol. 20 (1971):3295-3308; Gish, et. al., J. Med. Chem. 14 (1971):1159-1162; Aoshima, et al., Cancer Research 36 (1976):2762-2732 or fluorodeoxyuridine (Schwendener, et al., Biochem. Biophys. Res. Comm. 126 (1985):660-666) provide active drug for a period longer than would occur after administration of the parent drug.

However, such prodrugs do not selectively deliver the drug to tumor tissue; enhanced toxicity often accompanies enhanced antitumor efficacy (Schwendener, et al., Biochem. Biophy. Res. Comm. 126 (1985):660-666).

Like 5FU and Ara-C, the size of the dose of other antineoplastic nucleoside analogs (including but not limited to fluorouracil arabinoside, mercaptopurine riboside, arabinosyl adenine, or fluorodeoxyuridine) or their prodrugs that is administered is limited by toxicity, reducing the potential efficacy that would be obtained if higher concentrations could be attained near tumor cells.

Previous suggestions for targeted prodrugs of antineoplastic nucleoside analogs are unsatisfactory. Bagshawe, et al., Patent Application WO 88/07378, proposed that the corresponding nucleotides of antineoplastic nucleosides could be converted back to the nucleoside with an appropriate enzyme; Senter, et al., Patent Application EP 88112646, similarly suggest the use of fluorouridine monophosphate to be activated by the enzyme

alkaline phosphatase conjugated to an antibody that binds to a tumor cell surface antigen. These proposals fail to take into account the high and ubiquitous activity of enzymes which convert nucleotides to nucleosides (e.g., 5'nucleotidase) in blood and tissues. Nucleotides (nucleoside phosphates) are therefore not useful for targeted delivery of antineoplastic nucleoside analogs.

b. Alkylating Agents

Nitrogen mustard alkylating agents are an important class of antineoplastic drugs. Examples of antineoplastic alkylating agents with clinical utility are: cyclophosphamide, melphalan, chlorambucil, or mechlorethamine. These agents share, as a common structural feature, a bis-(2-chloroethyl) grouping on a nitrogen which can alkylate and thereby damage nucleic acids, proteins, or other important cellular structures. The cytotoxic activity of alkylating agents is less dependent upon the cell cycle status of their targets than is the case for antimetabolites that affect nucleic acid synthesis. For this reason, the cytotoxicity of alkylating agents can be less selective for rapidly dividing cells (e.g., many tumors) relative to normal tissues, but on the other hand, it is more completely effective against populations of cells that are not synchronized in their cell cycles.

Previous attempts at designing targeted prodrugs of nitrogen mustard compounds have been unsuccessful. Bagshawe, et al., Patent Application WO 88/078378, disclose benzoic acid nitrogen mustard glutamides as prodrugs which are only 5 to 10 fold lower in toxicity than the corresponding activated drugs; these authors themselves state that for clinical use, the prodrug must be at least 100 times less toxic than the drug.

Kerr, et al., Cancer Immunol. Immunother. 31 (1990):202-206, disclose melphalan-N-p-hydroxyphenoxyacetamide (an amide derivative of melphalan) as a potential prodrug to be activated with the enzyme penicillin-V-amidase (PVA). While this prodrug was in fact more than 100 fold less toxic than melphelan to particular cell lines in culture, pretreatment of cells

with an antibody-PVA conjugate failed to enhance the toxicity of the prodrug because PVA hydrolyzed the phenoxyacetamide bond of the prodrug too slowly to generate a toxic level of drug.

c. Other Antineoplastic Agents

The anthracyclines, daunorubicin, and doxorubicin, are widely used antitumor agents that exert a number of biochemical effects that contribute to both therapeutic and toxic effects of the drugs. One of the primary mechanisms of the drugs is to intercalate DNA and to destroy gene replication in dividing cells. Doxorubicin is effective in acute leukemias and malignant lymphomas. It is very active in a number of solid tumors. Together with cyclophosphamide and cisplatin, doxorubicin has considerable activity against carcinoma of the ovary. It has been used effectively in the treatment of osteogenic sarcoma, metastatic adenocarcinoma of the breast, carcinoma of the bladder, neuroblastoma and metastatic thyroid carcinoma. The myocardial toxicity of doxorubicin limits the dose of this drug that a patient may receive.

Catalytic Proteins

a. Enzymes

The prior art discloses the use of non-mammalian enzymes conjugated to targeting antibodies in order to activate the prodrug selectively at tumor sites (e.g., carboxypeptidases described in Bagshawe, et al., Patent Application WO 88/078378; Penicillin-V amidase described in Kerr, et al., Cancer Immunol. Immunother., 31 (1990):202-6; and β-lactamase described in Eaton, et al., Patent Application EP 90303681.2). Non-mammalian enzymes will generally be antigenic, and will thus be useful only for short term use or perhaps only a single use, due to the formation of neutralizing antibodies or the induction of undesirable immune responses.

In the cases where mammalian enzymes have been proposed e.g., alkaline phosphatase (Senter, et al., Patent Application EP 88112646), no provision has been made to obviate the problem of endogenous human enzymes activating the prodrug. Enzymes from different

species of mammals will also present problems due to antigenicity. In addition, some proposed prodrug-activating enzymes, e.g., neuraminidase (Senter, et al., Patent Application EP 88/112646) could cause serious damage to the organism to which they are administered; neuraminidase removes the sialic acid residue at the terminus of oligosaccharides on glycoproteins (important components of erythrocyte membranes, for example), exposing galactose residues which mark such glycoproteins for rapid degradation in the liver. Due consideration of the situation *in vivo* is necessary for practical implementation of the strategy of targeted activation of prodrugs of antineoplastic agents in embodiments suitable for use in humans.

b. Catalytic Antibodies

The manner in which catalytic antibodies carry out chemical reactions on substrates (or antigens) is essentially governed by the same theoretical principles that describe how enzymes carry out chemical reactions. See U. S. Patent 4,888,281, hereby incorporated by reference, which describes the catalysis of chemical reactions by antibodies. For most chemical transformations to occur, substantial activation energy is required to overcome the energy barrier that exists between reactant and product. Enzymes catalyze chemical reactions by lowering the activation energy required to form the short-lived unstable chemical species found at the top of the energy barrier, known as the transition state (Pauling, L., Am. Sci. 36 (1948):51; Jencks, W. P., Adv. Enzymol. 43 (1975):219). Four basic mechanisms are employed in enzymatic catalysis to stabilize the transition state, thereby reducing the free energy of activation. First, general acid and base residues are often found optimally positioned for participation in catalysis within catalytic active sites. A second mechanism involves the formation of covalent enzyme-substrate intermediates. Third, model systems have shown that binding reactants in the proper orientation for reaction can increase the "effective concentration" of reactants by at least seven orders of magnitude (Fersht, A. R., et al., Am. Chem. Soc. 90 (1968):5833) and therefore greatly reduce the entropy of a chemical

reaction. Finally, enzymes can convert the energy obtained upon substrate binding to distort the reaction towards a structure resembling the transition state.

Drawing upon this understanding of enzymatic catalysis, several antibodies with catalytic activity have been induced by immunization and isolated (Powell, M. J., et al., Protein Engineering 3 (1989):69-75). One approach for inducing acid or base residues into the antigen binding site is to use a complementary charged molecule in the immunogen. This technique proved successful for elicitation of antibodies with a hapten containing a positively-charged ammonium ion (Shokat, et al., Chem. Int. Ed. Engl. 27 (1988):269-271). Several of these monoclonal antibodies catalyzed a beta-elimination reaction.

In another approach, antibodies are elicited to stable compounds that resemble the size, shape, and charge of the transition state of a desired reaction (i.e., transition state analogs). See U. S. Patent 4,792,446 and U. S. Patent 4,963,355 which describe the use of transition state analogues to immunize animals and the production of catalytic antibodies. Both of these patents are hereby incorporated by reference.

Examples of catalytic antibodies that are able to accelerate reactions by stabilizing the transition state structure and/or enhancing the "effective concentration" of reactants are discussed below.

1. Esterases

The mechanism of ester hydrolysis involves a charged transition state whose electrostatic and shape characteristics can be mimicked by a phosphonate structure. Immunization of a mouse with a nitrophenyl phosphonate ester hapten-protein conjugate led to the isolation of monoclonal antibodies with hydrolytic activity on methyl-p-nitrophenyl carbonate (Jacobs, et al., J. Am. Chem. Soc. 109 (1987):2174-2176). An antibody against a similar transition state analog could hydrolyze its ester substrate in an organic matrix (Durfor, et al., J. Am.

Chem. Soc. 110 (1988):8713-8714). A substantial catalytic rate increase was reported for an antibody raised by immunization with a dipicolinic phosphonate ester (Tramontano, et al., J. Am. Chem. Soc. 110 (1988):2282). The antibody hydrolyzed 4-acetamidophenyl esters with a kcat of 20 s^{-1}, which was 6 million times faster than the rate constant for uncatalyzed ester decomposition. A recent report on the stereospecific cleavage of alkyl esters containing D-phenylalanine versus L-phenylalanine by monoclonal antibodies raised against phosphonate esters adds further credence to the use of phosphonate esters to elicit catalytic esterase monoclonal antibodies (Pollack, et al., J. Am. Chem. Soc. 111 (1989):5961-5962).

2. Peptidases/Amidases

Several ways of designing a transition state analog to mimic the transition state for a peptidase or amidase have been described. One report discussed the use of an aryl phosphonamidate transition state analog to produce an antibody that could cleave an aryl carboxamide (Janda, et al., Science 241 (1988):1188-1191). Another scheme for production of peptidases utilized a metal complex cofactor linked to a peptide (Iverson, et al., Science 243 (1989):1184-1188). Although the site of cleavage was not predicted by this method, further studies may allow for site-directed cleavage. Naturally occuring proteolytic antibodies have been found in humans (Paul, et al., Science 244 (1989):1158-1162). The antibodies were originally discovered in a subpopulation of asthma patients. One antiserum preparation cleaved a 28 amino acid polypeptide, vasoactive intestinal peptide (VIP) at one specific cleavage site.

3. Other Catalytic Antibodies

Other reactions which monoclonal antibodies have catalyzed are: a Claisen rearrangement (Jackson, et al., J. Am. Chem. Soc. 110 (1988):4841-4842; Hilvert, et al., Proc. Natl. Acad. Sci. USA 85 (1988):4953-4955; Hilvert, et al., J. Am. Chem. Soc. 110 (1988):5593-5594), redox reactions (Shokat, et al., Angew. Chem. Int. Ed. Engl. 27 (1989):269-271), photochemical cleavage of a thymine dimer (Cochran, et al., J. Am. Chem. Soc. 110 (1988):7888-7890) stereospecific transesterification rearrangements (Napper, et al., Science

237 (1987):1041-1043) and a bimolecular amide synthesis (Benkovic, et al., Proc. Natl. Acad. Sci. USA 85 (1988):5355-5358; Janda, et al., Science 241 (1988):1188-1191).

Objects of the Invention

It is an object of the invention to provide novel prodrugs of cytotoxic chemotherapeutic agents.

It is an object of the invention to provide methods for localizing formation or delivery of cytotoxic chemotherapeutic agents to or near tumors.

It is an object of the invention to provide prodrugs with a high drug/prodrug cytotoxicity ratio, which are essentially stable to endogenous mammalian enzymes and which are activated by targeted catalytic proteins of the invention.

It is an object of the invention to provide methods for localizing formation or delivery of cytotoxic chemotherapeutic agents to or near tumors to overcome the problems of 1) toxicity toward normal tissues and 2) reduced antitumor efficacy due to utilization or inactivation of the drugs at non-tumor sites.

It is an object of the invention to provide methods for selective targeting of active alkylating species to tumor cells.

It is an object of the invention to reduce systemic drug toxicity through specific tumor site activation of prodrugs using tumor-specific antibody binding and prodrug activation.

It is an object of the invention to provide prodrugs that are stable to mammalian enzymes, ensuring minimal drug activation or degradation outside the targeted tumor cells.

Summary of the Invention

These and other objects of the invention are achieved by prodrug compounds, and haptens which are used to produce antibodies capable of cleaving the protective groups from the prodrugs. In the prodrug compounds, a protective moiety lends stability to the compound, i.e., compounds of the invention are resistant to conversion to active drugs after administration, and substantially reduce the toxicity of the prodrug relative to the drug advantageously by at least one hundred fold.

The haptens of the invention are capable of producing catalytic antibodies by *in vitro* techniques followed by protein engineering of the antibodies found to be specific for the haptens, e.g., by random or site-directed mutagenesis, or by eliciting immune responses in mice or other hosts. The antibodies so-produced are capable of cleaving the protective moiety from the drug by esterase, amidase, hydrolase or glycosidase activity.

In the preferred embodiments of the invention, prodrug compounds are identified which meet the desired stability and toxicity characteristics and haptens are identified which have structural similarity to the same formula as the prodrug compounds and are capable of producing antibodies which catalytically cleave the drug from the residue of the compound.

One embodiment of the invention includes:

an immunoconjugate for treatment of specific cell populations comprising:

(a) a moiety capable of binding to an epitope of a specific cell population, and

(b) a catalytic antibody moiety capable of activating a prodrug.

Novel immunoconjugates include catalytic antibody moieties which activate novel prodrugs of the subject invention or prodrugs of the prior art.

The term moiety as used herein with reference to immunoconjugates means the whole antibody, enzyme or targeting protein, or active fragment thereof.

The invention also includes a therapeutic combination comprising:

(a) a novel prodrug of the subject invention, and

(b) an immunoconjugate comprising:

(i) a moiety capable of binding to an epitope of a specific cell population, and

(ii) a catalytic antibody moiety or enzyme moiety capable of activating said novel prodrug of the subject invention.

The invention also includes a therapeutic combination comprising:

(a) a prodrug of the prior art, and

(b) an immunoconjugate comprising:

(i) a moiety capable of binding to an epitope of a specific cell population, and

(ii) a catalytic antibody moiety capable of activating said prodrug of the prior art.

The invention also includes methods for treating various disease conditions by delivering a drug to a specific cell population such as a tumor. A targeting compound, e.g., an antibody, to which a catalytic antibody of the invention or fragment thereof is conjugated, is administered and permitted to become localized at the cell population. Thereafter, the prodrug is administered and is cleaved (i.e. activated) at the cell population to deliver the drug. Thus, included in the invention is a method of treating a condition of a specific cell population (e.g. cancer) comprising the steps of:

(a) administering an immunoconjugate comprising:

(i) a moiety capable of binding to an epitope of a specific cell population, and

(ii) a catalytic antibody moiety or enzyme moiety capable of activating a novel prodrug of the subject invention;

(b) permitting said immunoconjugate to become localized at said cell population; and

(c) administering a novel prodrug of the subject invention which is activated by said immunoconjugate.

Also included is a method of treating a condition of a specific cell population (e.g. cancer) comprising the steps of:

(a) administering an immunoconjugate comprising:

(i) a moiety capable of binding to an epitope of a specific cell population, and

(ii) a catalytic antibody moiety capable of activating a prodrug of the prior art;

(b) permitting said immunoconjugate to become localized at said cell population; and

(c) administering a prodrug of the prior art which is activated by said immunoconjugate.

A further embodiment of the invention is a method for identifying an antibody capable of activating a prodrug of interest comprising the steps of:

(i) immunizing a host with a hapten selected to elicit an antibody capable of activating the prodrug of interest and which is also capable of inactivating an antibiotic;

(ii) isolating recombinant genes coding for said antibody;

(iii) inserting the genes coding for said antibody into bacteria;

(iv) culturing said bacteria in a medium containing the antibiotic;

(v) selecting those bacteria which survive;

(vi) isolating antibody genes from the surviving bacteria;

(vii) expressing the antibody genes to produce sufficient quantity of antibody to characterize the antibody; and

(viii) screening the antibody for the capability of activating the prodrug of interest.

A further embodiment of the invention is a method for identifying an antibody capable of activating a prodrug of interest comprising the steps of

(i) immunizing a host with a hapten selected to elicit an antibody capable of activating the prodrug of interest;

(ii) isolating recombinant genes coding for said antibody;

(iii) inserting the genes coding for said antibody into bacteria;

(iv) culturing said bacteria in a medium containing thymidine derivatized by the same promoiety as the prodrug of interest;

(v) selecting those bacteria which survive;

(vi) isolating antibody genes from the surviving bacteria;

(vii) expressing the antibody genes to produce sufficient quantity of antibody to characterize the antibody; and

(viii) screening the antibody for the capability of activating the prodrug of interest.

A still further embodiment of the invention is a method of screening for antibodies capable of catalyzing the conversion of substrate to product comprising the steps of:

(i) raising antibodies against a hapten,

(ii) immobilizing said antibodies,

(iii) adding a substrate to said antibodies, and

(iv) identifying antibodies capable of catalyzing the conversion of substrate to product.

Optionally, after step (i) is the step of selecting antibodies which bind said hapten.

A further embodiment of the invention is a method of screening for cells expressing an antibody capable of catalyzing a reaction comprising the steps of:

(i) plating out cells auxotrophic for a compound and containing antibody genes, in a culture medium containing a proform of said compound; and

(ii) selecting those cells which survive which express an antibody capable of activating said proform to release said compound.

A further embodiment of the invention is a method of screening for cells expressing an antibody capable of activating a prodrug comprising the steps of:

(i) plating out thymidine dependent cells containing antibody genes in a culture medium containing a prodrug where said drug is thymidine; and

(ii) selecting those cells which survive which express an antibody capable of activating said prodrug to form thymidine.

A further embodiment of the invention is a method of screening for cells expressing an antibody capable of catalyzing a reaction comprising the steps of:

(i) plating out cells containing antibody genes in a culture medium containing a toxin; and

(ii) selecting those cells which survive which express an antibody capable of inactivating said toxin.

A still further embodiment of the invention is a method of screening for cells expressing an antibody capable of activating a prodrug comprising the steps of:

(i) plating out bacteria cells containing antibody genes in a culture medium containing an antibiotic; and

(ii) selecting those bacteria cells which survive which express an antibody capable of inactivating said antibiotic.

Another embodiment of the invention is a method of synthesizing a bispecific antibody comprising the steps of:

(i) expressing a gene having a sequence selected from the group consisting of:

VH antibody 1-S-VL antibody 1-S-VL antibody 2-S-VH antibody 2;

VH antibody 1-S-VL antibody 1-S-VH antibody 2-S-VL antibody 2;

VL antibody 1-S-VH antibody 1-S-VL antibody 2-S-VH antibody 2;

VL antibody 1-S-VH antibody 1-S-VH antibody 2-S-VL antibody 2;

wherein -S- is a linker sequence; and

(ii) isolating said bispecific antibody.

Antibody 1 is an antibody capable of binding to an epitope of a specific cell, and antibody 2 is a catalytic antibody or vice versa.

A further embodiment of the invention is a method of synthesizing a bispecific antibody comprising the steps of:

(i) expressing a gene having the sequence:

VL antibody 1-S-VH antibody 2, and

(ii) expressing a gene having the sequence:

VH antibody 1-S-VL antibody 2,

(iii) combining the products of steps (i) and (ii), and

(iv) isolating said bispecific antibody,

wherein -S- is a linker sequence.

A still further embodiment of the invention is a method of synthesizing a bispecific antibody comprising the steps of:

(i) expressing a gene having the sequence;

VL antibody 2-S-VH antibody 1, and

(ii) expressing a gene having the sequence:

VH antibody 2-S-VL antibody 1,

(iii) combining the products of steps (i) and (ii), and

(iv) isolating said bispecific antibody,

wherein -S- is a linker sequence.

Brief Description of the Drawings

Figure 1a shows the preparation of linear trimethylbenzoyl- and trimethoxybenzoyl-5-fluorouridine prodrugs, Compound 1a and 1b.

Figure 1b shows the preparation of the hapten of the prodrug in Example 1a, the linear phosphonate of trimethoxybenzoate-5-fluorouridine, Compound 4.

Figure 1c shows the preparation of the prodrug, 5'-0-(2,6-dimethoxybenzoyl)-5-fluorouridine, Compound 1c.

Figure 1d shows the preparation of the hapten of the prodrug in Example 1a: the linear phosphonate of trimethylbenzoate-5-fluorouridine, Compound 4a.

Figure 2a shows the preparation of the prodrug, intramolecular trimethoxybenzoate-5-fluorouridine, Compound 10.

Figure 2b shows the preparation of the hapten of prodrug in Example 2a: the cyclic phosphonate of trimethoxybenzoate-5-fluorouridine, Compound 15.

Figure 3 shows the preparation of experimental prodrug, galactosyl cytosine β-D-arabinofuranoside, Compound 19.

Figure 4 shows the preparation of experimental prodrug, galactosyl 5-fluorouridine, Compound 24.

Figure 5a shows the prepartation of the precursor to the hapten of the prodrugs in Examples 3 and 4, Compound 25.

Figure 5b shows the preparation of the hapten of the prodrugs in Examples 3 and 4, Compounds 30a and 30b.

Figure 5c shows the alternative preparation of the hapten of the prodrugs in Examples 3 and 4, Compounds 30a and 30b.

Figure 6 shows the preparation of the experimental prodrug, aliphatic diethyl acetal protected aldophosphamide, Compound 38.

Figure 7 shows the preparation of the guanyl hapten of the experimental prodrug, aliphatic diethyl acetal protected aldophosphamide, Compound 43.

Figure 8a shows the preparation of the anhydride intermediate, Compound 45, for the synthesis of intramolecular enol trimethoxybenzoate phosphamide prodrug.

Figure 8b shows the preparation of the Prodrug, intramolecular enol trimethoxybenzoate phosphamide, Compound 50.

Figure 8c shows the preparation of the intramolecular enol trimethoxybenzoate phosphamide hapten, Compound 57.

Figure 9 shows the comparison of AraC and galactosyl-AraC prodrug on Colo cells.

Figure 10 shows the comparison of AraC and galactosyl-AraC prodrug on Lovo cells.

Figure 11 shows the site specific activation of galactosyl-AraC prodrug on CEA antigen positive cells.

Figure 12 shows the activity of galactosyl-AraC prodrug on CEA antigen negative cells.

Figure 13 shows the white blood cell response to drug and prodrug.

Figure 14 shows the segmented neutrophil response to drug and prodrug.

Figure 15 shows the platelet response to drug and prodrug.

Figure 16 shows the lymphocyte response to drug and prodrug.

Figure 17 shows the red blood cell response to drug and prodrug.

Figure 18 shows the comparison of 5' fluorouridine and galactosyl-5' fluorouridine prodrug on CEA antigen negative Colo cells.

Figure 19 shows the site specific activation of 5' fluorouridine prodrug on CEA antigen positive Lovo cells.

Figure 20 shows the activity of 5' fluorouridine prodrug on CEA antigen negative Colo cells.

Figure 21 shows the comparison of 5' fluorouridine and galactosyl-5' fluorouridine prodrug on total leukocytes in mice.

Figure 22 shows the comparison of 5' fluorouridine and galactosyl-5' fluorouridine prodrug on red blood cells in mice.

Figure 23 shows the comparison of 5' fluorouridine and galactosyl-5' fluorouridine prodrug on total neutrophils in mice.

Figure 24 shows the comparison of 5' fluorouridine and galactosyl-5' fluorouridine prodrug on total lymphocytes in mice.

Figure 25 shows the comparison of 5' fluorouridine and galactosyl-5' fluorouridine prodrug on total bone marrow cellularity in mice.

Figure 26 shows the preparation of the intermediate of the prodrugs in Examples 16 and 20 and of the haptens of the prodrugs in Examples 18 and 22, the (thiazolyl)iminoacetic ester, Compound 60.

Figure 27 shows the preparation of the prodrug, the 5-fluorouridine substituted β-lactam, Compound 68.

Figure 28 shows the preparation of the intermediate of the hapten of the prodrug in Example 16, the 5-alkynylated uridine, Compound 74.

Figure 29 shows the preparation of the intermediate of the hapten of the β-lactam prodrug, Compound 79.

Figure 30 shows the preparation of the hapten of the prodrug in Example 16, the cyclobutanol substituted 5-fluorouridine, Compound 81.

Figure 31 shows the preparation of the intermediate of the prodrug in Example 20, the 5-fluorouridine 5'-O-aryl ester, Compound 85.

Figure 32 shows the preparation of the prodrug, the β-lactam substituted by a 5'-O-aroyl-5-fluorouridine, Compound 90.

Figure 33 shows the preparation of the intermediate of the hapten in Example 22, the 5-alkynylated uridine 5'-O aryl ester, Compound 92.

Figure 34 shows the preparation of the hapten of the prodrug in Example 20, the cyclobutanol substituted by a 5'-O-aroyl uridine, Compound 100.

Figure 35 shows the preparation of the adriamycin prodrug, aroylamide, Compound 103.

Figure 36 shows the preparation of the hapten of the adriamycin prodrug, in Example 23, the phosphate of the aroylamide of adriamycin, Compound 104.

Figure 37 shows the preparation of the hapten of the prodrug in Example 23, the aroyl sulphonamides of adriamycin, Compound 106.

Figure 38 shows the preparation of melphalan aroylamide prodrugs, Compound 109.

Figure 39 shows the preparation of the hapten of the prodrug in Example 25. The sulphonamide of the aroylamide of melphalan, Compound 110.

Figure 40 shows the preparation of the prodrug, tetrakis(2-chloroethyl)aldophosphamide diethyl acetal, Compound 112.

Figure 41 shows the preparation of the hapten of the prodrug in Example 31: The trimethylammonium salt analog of tetrakis(2-chloroethyl)aldophosphamide diethyl acetal, Compound 119.

Figure 42 shows the preparation of the hapten of the prodrug in Example 31: The dipropylmethylammonium salt analog of tetrakis(2-chloroethyl)aldophosphamide diethyl acetal, Compound 121.

Figure 43 shows the preparation of the prodrug, intramolecular bis(2-hydroxyethoxy)benzoate-5-fluorouridine, Compound 128.

Figure 44 shows the preparation of the hapten of the prodrug in Example 34: The cyclic phosphonate analog of bis(2-hydroxyethoxy)benzoate-5-fluorouridine, Compound 137.

Figure 45 shows the preparation of the prodrug, intramolecular bis(3-hydroxypropyloxy)benzoate-5-fluorouridine, Compound 138.

Figure 46 shows the preparation of the hapten of the prodrug in Example 36: The cyclic phosphonate analog of bis(3-hydroxypropyloxy)benzoate-5-fluorouridine, Compound 139.

Figure 47 shows the preparation of the prodrug: 5'-O-(2,4,6-trimethoxybenzoyl)-5-fluorouridine, Compound 141.

Figure 48a shows the preparation of the hapten of the prodrug in Example 38: The pyridinium alcohol-substituted analog of uridine, Compound 149.

Figure 48b shows the preparation of the hapten of the prodrug in Example 38: The pyridinium alcohol-substituted analog of uridine, Compound 149.

Figure 49 shows the preparation of the hapten of the prodrug in Example 38: The linear phosphonate of 5'-O-(2,4,6-trimethoxybenzoyl)-5-fluorouridine, Compound 152.

Figure 50 shows the preparation of the hapten for the prodrug in Example 1a: The linear phosphonate of 5'-O-(2,6-dimethoxybenzoyl)-5-fluorouridine, Compound 155.

The invention, as well as other objects, features, and advantages thereof, will be understood more clearly and fully from the following detailed description when read with reference to the accompanying figures which illustrate the results of the experiments discussed in the examples below.

Detailed Description of the Invention

The invention provides specific methods for converting a variety of cancer chemotherapy drugs to substantially non-toxic prodrugs which are stable to endogenous enzymes, but which can be activated in or near tumors by prior administration of tumor-selective agents such as receptor-binding ligands, analogs which bind to tumor associated enzymes, and antibodies conjugated to or otherwise physically connected to a protein catalyst which converts the prodrugs to active cytotoxic agents. The catalytic protein is 1) a catalytic antibody, 2) an exogenous (or non-mammalian) enzyme, or 3) an endogenous (or mammalian) enzyme with low endogenous activity in the compartments to which the prodrug has access after administration. Such a system permits formation of relatively high concentrations of active agent localized at the tumor site(s) while also reducing systemic exposure to the drugs.

The invention provides prodrugs with a high drug/prodrug cytotoxicity ratio, which are essentially stable to endogenous mammalian enzymes and which are activated by targeted catalytic proteins of the invention.

The invention provides compounds and methods for preparing suitable prodrugs of antineoplastic nucleoside analogs that are substantially non-toxic *in vivo* until activated by a catalytic protein of the invention.

In designing prodrugs of cytotoxic agents for targeted activation, it is important that the prodrug substituents impart two properties to the drug: (1) that they are relatively stable after

administration, and are therefore, relatively non-toxic; and (2) that they are specifically activatable. Furthermore, the prodrug substituents should not be toxic to the organism after cleavage by the catalytic protein.

In the invention, prodrugs of antineoplastic agents are made by attaching appropriate substituents, described below, to antineoplastic drugs. Substituents are chosen which render the parent drug relatively non-toxic and which are relatively resistant to removal by endogenous enzyme activity, but which are removed (yielding active drug) by the catalytic proteins of the invention.

Preferred substituents on the prodrug and on haptens for the prodrug are H, alkyl with 1-10 carbon atoms, alkoxy with 1-10 carbon atoms, monocyclic aromatic alkene with 1-10 carbon atoms, hydroxyl, hydroxyalkyl, hydroxyalkoxy, aminoalkyl, thioalkyl, amino, alkylamino, alkylphosphonate, alkylsulfonate, alkylcarboxylate, alkylammonium cyclicalkyl, substituted cyclicalkyl, or cyclicalkyl substituted with at least on heteroatom in the ring.

The substituents on the prodrug and on haptens for the prodrug comprising alkyl, alkenyl, alkynyl, substituted alkyl, alkenyl and alkynyl, hydroxyalkyl, hydroxyalkoxy, aminoalkyl, thioalkyl, alkylamino, alkylphosphonate, alkylsulfonate, alkylcarboxylate, alkylammonium, cyclicalkyl, substituted cyclicalkyl, and cyclicalkyl substituted with at least one heteroatom in the ring preferably have 1-10 carbon atoms in the carbon chain or ring.

Wherein the substituents on the prodrug and on haptens for the prodrug are substituted, the preferred substituents are -OH, alkyl, chloro, fluoro, bromo, iodo, $-SO_3$, aryl, -SH, -(CO)H, -(CO)OH, ester groups, ether groups, alkenyl, alkynyl, -CO-, $-N_2+$, cyano, epoxide groups and heterocyclic groups.

Preferred heteroatoms in the prodrug and in haptens for the prodrug are phosphorus, sulfur, nitrogen, and oxygen. The substituents on the prodrug and on haptens for the prodrug which contain heteroatoms preferably contain one or more heteroatoms.

Preferred counterions (anions) for positively charged quaternary amines in the prodrug and in haptens for the prodrug are halogens, acetate, methane sulfonate, para-toluene sulfonate, and trifluoromethane sulfonate.

Catalytic proteins, and especially catalytic antibodies, most easily catalyze reactions with relatively low activation energies. Reactions that are known to be catalyzed or accelerated by antibodies include ester cleavage, Claisen rearrangement, redox reactions, stereospecific transesterification rearrangements, and amide or peptide cleavage.

Catalytic antibodies, as well as enzymes, catalyze chemical reactions by lowering the activation energy required to form the short-lived, unstable transition state. Catalytic antibodies which stabilize or enhance the formation of the transition state are produced by generating antibodies to stable analogs of the prodrugs that resemble the size, shape, and charge of the transition state of the substituent-cleavage reaction. For example, transition state analogs of ester-cleavage reactions (haptens) are prepared by substituting a stable phosphonate or sulfonate group for the normal carbonyl group.

The transition state analogs are typically used as haptens for eliciting antibodies with catalytic activity toward prodrugs of the invention. As such, their structure generally includes a linker arm for attachment to a protein carrier. Thus, the moiety of the hapten corresponding to the drug in the prodrug is typically an analog of the original drug, differing in the presence of a covalently-attached linker arm terminating in a group which can be attached to a protein. In some embodiments of the invention, the linker arm is attached to the moiety of the hapten

corresponding to the prodrug substituent (e.g., the substituted benzoate portion of an ester prodrug of a nucleoside analog) of the prodrug.

In some transition state analogs, the drug-like moiety in the hapten is also optionally modified to provide structural similarity to the transition state for the prodrug-activation reaction. For example, in drugs bearing a hydroxyl group through which the drug is attached to its prodrug moiety, the oxygen of attachment (which is normally part of the drug molecule) is replaced by -NH-, -CH_2-, or -S- in the corresponding hapten.

Furthermore, the drug-like moiety in the hapten is also optionally modified to give it structural rigidity in a conformation favorable for eliciting antibodies with catalytic activity toward the corresponding prodrug. In most cases, however, the moiety of the transition-state analog corresponding to the drug portion of the prodrug has a substantial structural similarity to the original drug. Examples of haptens made from analogs of the drug moieties of their corresponding prodrugs are shown below.

A preferred drug-like moiety in the hapten is an analog of 5-fluorouridine which is substituted in the 5-position by a moiety comprising -C-C-$(CH_2)_n$NHCBz or $(CH_2)_nNH_2$, where n is an integer between 1 and 10, and CBz is carbobenzyloxy.

Another preferred drug-like moiety in the hapten is an analog of phosphoramide mustard [R'OP(O) (R")N$(CH_2CH_2CL)_2$]), wherein R' and R" are the same or different and independently from one another are H, alkyl with 1-10 carbon atoms, monocyclic aromatic, alkene with 1-10 carbon atoms, hydroxyl, hydroxyalkyl, hydroxyalkoxy, aminoalkyl, thioalkyl, amino, alkylamino, alkylphosphonate, alkylsulfonate, alkylcarboxylate, alkylammonium, cyclicalkyl, substituted cyclicalkyl, or cyclicalkyl substituted with at least one heteroatom in the ring. A preferred embodiment is a drug-like moiety in the hapten

wherein R' is alkylammonium salt; and where R" is a substituted cyclicalkyl wherein the cyclicalkyl is substituted with two heteroatoms in the ring.

Substantial esterase activity is present and ubiquitous in mammalian tissues. This activity is relatively nonspecific, cleaving ester bonds in a large variety of compounds. However, some classes of prodrugs of the invention, e.g., substituted aromatic esters of nucleoside analogs, have ester substituents which are relatively resistant to endogenous mammalian esterase activity.

Similar substituted aromatic esters and other prodrug substituents of the invention are useful for preparing prodrugs of a variety of classes of antineoplastic agents with appropriate functional groups, including but not limited to nucleoside analogs and other antimetabolites, alkylating agents such as cyclophosphamide derivatives, intercalating agents such as doxorubicin or etoposide, spindle poisons such as vinca alkaloids, or other classes of cytotoxic drugs.

The prodrugs of the invention, which are relatively resistant to activation by endogenous mammalian enzymes, are activated by the catalytic proteins of the invention, e.g., catalytic antibodies (or active fragments thereof) prepared by raising antibodies to analogs of the transition states of the prodrug activation reactions.

The catalytic proteins of the invention are conjugated to, or otherwise physically associated with, a tumor-selective antibody, antibody fragment, or binding protein or analogs to tumor-associated proteins or tumor-selective receptor ligands. . This complex is typically administered prior to the prodrug, so that it is localized in or near cancer cells. The prodrug is then administered and cleaved by the catalytic protein, forming active antineoplastic drugs in or near tumors.

Below are described various prodrugs of the invention as well as transition state analogs corresponding to such prodrugs. Additionally described are the haptens which can be used to produce antibodies capable of cleaving the protective groups from the prodrugs.

Novel Prodrugs and Haptens of the Invention

Classes of Prodrug Substituents and Prodrug Activation Reactions

Within the broad category of catalytic antibody-mediated hydrolysis reactions, there are several classes of specific catalytic antibody-mediated catalytic reactions which are most suitable for use with appropriate prodrugs in order to effect their activation. Catalytic antibodies with the following types of activity are prepared and utilized:

A. Esterase - cleaves acyl substituents esterified to drugs

B. Amidase - cleaves acyl substituents attached to amino groups

C. Acetal hydrolase - hydrolyzes acetals (or ortho esters) to aldehydes (or acids)

D. Glycosidase - cleaves sugar substituents attached to drugs via a glycoside linkage.

Catalytic antibodies with these classes of activity are typically elicited by immunization of animals with haptens that mimic the transition state of the prodrug activation reactions. Prodrug substituents which are relatively stable to mammalian enzymatic activity are designed and utilized in creating transition state analogs which are in turn utilized to produce catalytic antibodies capable of activating the prodrugs. In cases where enzymes capable of activating prodrugs of the invention exist, they are optionally used for this purpose as an alternative to catalytic antibodies.

The prodrugs themselves are also optionally used to elicit antibodies with catalytic activity. Conversely, transition state analogs of prodrugs are also optionally useful as prodrugs or drugs. Typically, however, the compounds designated as prodrugs are utilized as such, and

the compounds designated below as transition state analogs are utilized as haptens for eliciting catalytic antibodies.

Prodrug substituents which are relatively stable to mammalian enzymatic activity and which are activated by the antibody-catalyzed reactions listed above include the following:

A. Prodrug Activation By Esterase Reaction

Steric hindrance from the substituents on the benzoate or acetate moieties inhibits their cleavage by endogenous esterase activity (see Example 27). Examples of these are as follows:

1. Substituted aromatic esters, e.g., substituted benzoate esters;
2. Substituted aromatic esters activated by an intramolecular nucleophilic attack on the ester carbonyl;
3. Di- or tri-substituted acetate esters; and
4. Di- or tri-substituted acetate esters activated by an intramolecular nucleophilic attack on the ester carbonyl.

Other ester substituents which are stable to mammalian enzyme activity and which are cleaved by catalytic antibodies are within the scope of the invention.

Transition state analogs for ester hydrolysis reactions typically have a phosphonate or sulfonate group in the place of the original carbonyl group, as described in more detail below.

B. Prodrug Activation By Amidase Reaction

Amides in general, and those listed below in particular, are relatively stable to mammalian enzyme activity.

1. Aromatic or substituted aromatic amides, e.g., benzoate or substituted benzoate amides;
2. Aromatic or substituted aromatic amides activated by an intramolecular nucleophilic attack on the amide carbonyl;
3. Formylamides;
4. Acetylamides;
5. Acetylamides activated by an intramolecular nucleophilic attack on the amide carbonyl; and
6. Monolactam hydrolysis.

Transition state analogs for amide hydrolysis reactions typically have a phosphonate or sulfonate group in the place of the original carbonyl group, as described in more detail below.

C. <u>Prodrug Activation By Acetal Hydrolysis Reaction</u>

Acetal prodrugs of antineoplastic agents are stable and relatively non-toxic (see Example 29). Examples of these are as follows:

1. Dialkyl acetals;
2. Ortho esters;
3. Diol acetals, e.g., sugar-substituted acetals; and
4. Diol ortho esters.

Transition state analogs for acetal hydrolysis reactions typically have an amidine or guanidine group replacing the acetal group in the original prodrug.

D. <u>Prodrug Activation By Glycosidase Reaction</u>

Glycosyl derivatives of the invention are stable and relatively non-toxic (see Example 28). Examples of these are as follows:

1. Hexopyranose conjugated to drug hydroxyl group via the anomeric position of the sugar.
2. Hexofuranose conjugated to drug hydroxyl group via the anomeric position of the sugar.

Transition state analogs for glycosidase reactions typically have amino groups replacing the anomeric and ring oxygen atoms of the sugar.

* * * * * * * *

The antineoplastic agents utilized and derivatized in the invention contain hydroxyl groups or primary amino groups; the antineoplastic drugs are therefore represented in the compound descriptions below as XQH where Q is -O- or -NH-. X, as utilized in the compound description is the dehydroxy or deamino radical of the original drug. The moieties corresponding to the drug radical X in the transition state analogs are represented as X'. As described above, X' is typically an analog of the drug X, although X' may also be identical to the drug radical X. A preferred feature of X' is that it must bear sufficient structural similarity to the drug radical X so that the transition-state analog is capable of eliciting antibodies with catalytic activity toward the prodrug of XQH. Since the preferred site for catalysis is actually within the prodrug substituent, or at the juncture between substituent and drug, there is latitude in the structure of X'. Typically, however, X' will be very similar to X, generally differing in that X' contains a linker arm for joining the transition-state analog to a carrier protein such as bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH) for immunizing animals to elicit antibodies to the transition-state analog which have catalytic activity.

Esterase Catalysis

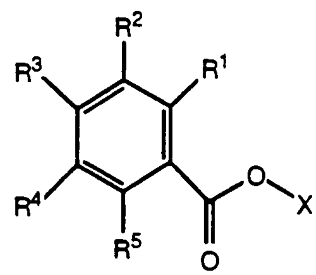
Novel compounds in accordance with the invention which are activated by esterase catalysis include compounds of the formulas set forth below:

A. Prodrug Activation By Esterase Reaction

1. Substituted aromatic esters, e.g., substituted benzoate esters

Substituted aromatic ester prodrug

Included in the invention is a substituted aromatic ester compound A1a having the formula:



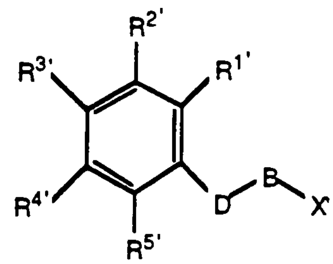
wherein X is a radical of the drug XOH. XOH is advantageously a cytotoxic drug such as an antineoplastic nucleoside analog (joined to the carboxyl moiety at the 3' and/or 5' position of the aldose ring), doxorubicin, or the enol form of aldophosphamide.

R^1, R^2, R^3, R^4 and R^5 are the same or different and are H, alkyl with 1-10 carbon atoms, alkoxy with 1-10 carbon atoms, monocyclic aromatic, alkene with 1-10 carbon atoms, hydroxyl, hydroxyalkyl, aminoalkyl, thioalkyl, amino, alkylamino, alkylphosphonate, alkylsulfonate, alkylcarboxylate, or alkylammonium, with the proviso that at least one of R^{1-5} are not H, and advantageously, R^1 or R^5 is not H.

The compound is not Ara-C-2,4,6-trimethyl benzoate, Ara-C-3,4,5-trimethoxy benzoate or Ara-C-2,6-dimethyl benzoate. However, Ara-C-2,4,6-trimethyl benzoate, Ara-C-3,4,5-trimethoxy benzoate or Ara-C-2,6-dimethyl benzoate are useful in the methods of treatment utilizing catalytic antibodies of the subject invention.

Hapten 1

Useful as a hapten as well as a prodrug is a compound A1b having the formula:



wherein X' is an analog of X of compound A1a, and X' is optionally linked to a carrier protein,

B is O, S, NH, or CH_2,

D is P(O)OH, SO_2, CHOH or SO (with any stereochemistry), if D is CHOH then B is CH_2, and

$R^{1'}$, $R^{2'}$, $R^{3'}$, $R^{4'}$ and $R^{5'}$ are the same or different, are optionally linked to a carrier protein and are H, alkyl with 1-10 carbon atoms, alkoxy with 1-10 carbon atoms, monocyclic aromatic, alkene with 1-10 carbon atoms, hydroxyl, hydroxyalkyl, aminoalkyl, thioalkyl, amino, alkylamino, alkylphosphonate, alkylsulfonate, alkylcarboxylate, or alkylammonium, with the proviso that at least one of $R^{1'-5'}$ are not H. Advantageously, $R^{1'}$ or $R^{5'}$ is not H.

Hapten 2

Included in this invention is a substituted aromtic compound A1a' having the formula:

wherein X is a radical of the drug XOH. XOH is advantageously a cytotoxin drug such as an antinucleoplastic nucleoside analog (joined ato B at the 3' and/or 5' position of the aldose ring), doxorubicin, or the enol form of aldeophosphamide:

Z is C or N;

B is O, S, NH or CH_2;

D is HOP(O), SO_2, CHOH or SO (with any stereochemistry);

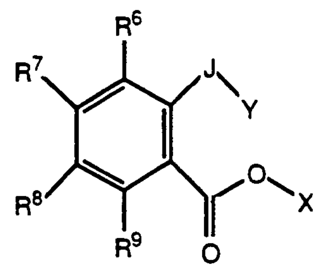
R^1, R^2, R^3, R^4 and R^5 are the same or different and are H, alkyl with 1-10 carbon atoms, alkoxy with 1-10 carbon atoms, monocyclic aromatic, alkene with 1-10 carbon atoms,

hydroxyl, hydroxyalkyl, hydroxyalkoxy, haloalkyl, aminoalkyl, thioalkyl, amino, alkylamino, alkylphosphonate, alkylsulfonate, alkylcarboxylate, or alkylammonium, with the proviso that at least one of R^{1-5} are not H, and advantageously, R^1 or R^5 is not H.

2. Substituted aromatic esters activated by an intramolecular nucleophilic attack on the ester carbonyl

Substituted aromatic ester prodrug

Included in the invention is a substituted aromatic ester compound A2a having the formula:



wherein X is a radical of the drug XOH. Advantageously, XOH is a cytotoxic drug such as an antineoplastic nucleoside analog (joined to the carboxyl moiety at the 3' and/or 5' position of the aldose ring), doxorubicin, or the enol form of aldophosphamide.

R^6, R^7, R^8, and R^9 are the same or different and are H, alkyl with 1-10 carbon atoms, alkoxy with 1-10 carbon atoms, monocyclic aromatic, alkene with 1-10 carbon atoms, hydroxyl, hydroxyalkyl, aminoalkyl, thioalkyl, amino, alkylamino, alkylphosphonate, alkylsulfonate, alkylcarboxylate, or alkylammonium. Advantageously, at least 1 of R^{6-9} is not H.

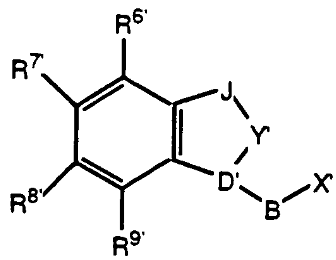
J is alkyl with 1-9 atoms in a linear configuration, alkyl with heteroatoms with one or more 1-9 atoms in a linear configuration which have substituents that are phenyl, alkyl, or alkyl with heteroatoms.

Y is OH, NH_2, NHR or SH where R is an alkyl, alkenyl or alkynyl optionally substituted by one or more substituents selected from the group consisting of -OH, chloro,

fluoro, bromo, iodo, $-SO_3$, aryl, -SH, -(CO)H, -(CO)OH, ester groups, ether groups, -CO-, cyano, epoxide groups and heteroatoms.

Hapten

Useful as a hapten as well as a prodrug is a compound A2b having the formula:



wherein X' is an analog of X of compound A2a, and X' is optionally linked to carrier protein,

B is O, S, NH, or CH_2,

D' is P(O), COH (with any stereochemistry), if D' is COH then B and Y' are CH_2,

Y' is O, NH, NR, S or CH_2 where R is an alkyl, alkenyl or alkynyl optionally substituted by one or more substituents selected from the group consisting of -OH, chloro, fluoro, bromo, iodo, $-SO_3$, aryl, -SH, -(CO)H, -(CO)OH, ester groups, ether groups, -CO-, cyano, epoxide groups and heteroatoms,

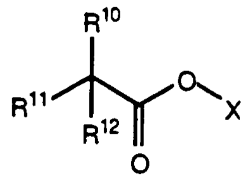
$R^{6'}$, $R^{7'}$, $R^{8'}$, and $R^{9'}$ are the same or different, are optionally linked to the carrier protein and are H, alkyl with 1-10 carbon atoms, alkoxy with 1-10 carbon atoms, monocyclic aromatic, alkene with 1-10 carbon atoms, hydroxyl, hydroxyalkyl, aminoalkyl, thioalkyl, amino, alkylamino, alkylphosphonate, alkylsulfonate, alkylcarboxylate, or alkylammonium. Advantageously, at least 1 of $R^{6'-9'}$ is not H.

J is alkyl with 1-9 atoms in a linear configuration, alkyl with one or more heteroatoms with 1-9 atoms in a linear configuration which have substituents that are phenyl, alkyl, or alkyl with one or more heteroatoms.

3. Di- or tri-substituted acetate esters

Di- or tri-substituted acetate ester prodrug

Included in the invention is a di or tri-substituted acetate ester compound A3a having the formula:



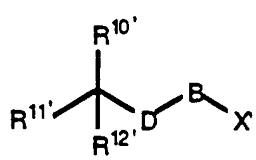
wherein X is a radical of the drug XOH. Advantageously, XOH is a cytotoxic drug such as an antineoplastic nucleoside analog (joined to the carboxyl moiety at the 3' and/or 5' position of the aldose ring), doxorubicin, or the enol form of aldophosphamide.

R^{10}, R^{11} and R^{12} are the same or different but at least two of them are not H, and are H or alkyl with 2 to 22 carbon atoms, alkyl with one or more heteroatoms, cycloalkyldiol, monocyclic aromatic, alkylphosphonate, alkylsulfonate, alkylcarboxylate, alkylammonium or alkene.

The compound is not Ara-C-diethyl acetate. However, Ara-C-diethyl acetate is useful in the methods of treatment utilizing catalytic antibodies of the subject invention.

Hapten

Useful as a hapten as well as a prodrug is a compound A3b having the formula:



wherein X' is an analog of X of A3a, and X' is optionally linked to carrier protein,

B is O, S, NH, or CH_2,

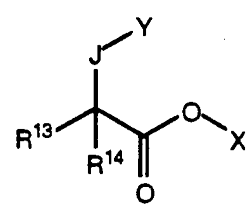
D is P(O)OH, SO_2, CHOH or SO with any stereochemistry, if D is CHOH then B is CH_2, and

$R^{10'-12'}$ which are optionally linked to the carrier protein, are the same or different but at least two of them are not H, and are H or alkyl with 2 to 22 carbon atoms, alkyl with one or more heteroatoms, cycloalkyldiol, monocyclic aromatic, alkylphosphonate, alkylsulfonate, alkylcarboxylate, alkylammonium or alkene.

4. Di- or tri-substituted acetate esters activated by an intramolecular nucleophilic attack on the ester carbonyl.

Di- or tri-substituted acetate ester prodrug

Included in the invention is a substituted acetate ester compound A4a having the formula:



wherein X is a radical of the drug XOH. Advantageously, XOH is a cytotoxic drug such as an antineoplastic nucleoside analog, doxorubicin, or the enol form of aldophosphamide.

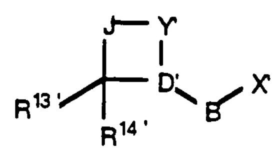
J is alkyl with 1-9 atoms in a linear configuration, alkyl with one or more heteroatoms with 1-9 atoms in a linear configuration which have substituents that are phenyl, alkyl, or alkyl with one or more heteroatoms,

Y is OH, NH_2, NHR or SH, where R is an alkyl, alkenyl or alkynyl optionally substituted by one or more substituents selected from the group consisting of -OH, chloro, fluoro, bromo, iodo, $-SO_3$, aryl, -SH, -(CO)H, -(CO)OH, ester groups, ether groups, -CO-, cyano, epoxide groups and one or more heteroatoms, and

R^{13-14} are the same or different but are not both H, and are H or alkyl with 2 to 22 carbon atoms, alkyl with one or more heteroatoms, cycloalkyldiol, monocyclic aromatic, alkylphosphonate, alkylsulfonate, alkylcarboxylate, alkylammonium or alkene.

Hapten

Useful as a hapten as well as a prodrug is a compound A4b having the formula:



wherein X' is an analog of compound A4a. and X' is optionally linked to a carrier protein,

B is O, S, NH, or CH_2,

D' is P(O), COH with any stereochemistry, if D' is COH, then B and Y' are CH_2,

Y' is O, NH, NR, S or CH_2 where R is an alkyl, alkenyl or alkynyl optionally subsituted by one or more substituents selected from the group consisting of -OH, chloro, fluoro, bromo, iodo, $-SO_3$, aryl, -SH, -(CO)H, -(CO)OH, ester groups, ether groups, -CO-, cyano, epoxide groups and one or more heteroatoms,

J is alkyl with 1-9 atoms in a linear configuration, alkyl with one or more heteroatoms with 1-9 atoms in a linear configuration which have substituents that are phenyl, alkyl, or alkyl with heteroatoms, and

$R^{13'-14'}$ which are optionally linked to a carrier protein are the same or different but at least two of them are not H, and are H or alkyl with 2 to 22 carbon atoms, alkyl with heteroatoms, cycloalkyldiol, monocyclic aromatic, alkylphosphonate, alkylsulfonate, alkylcarboxylate, alkylammonium or alkene.

Amidase Catalysis

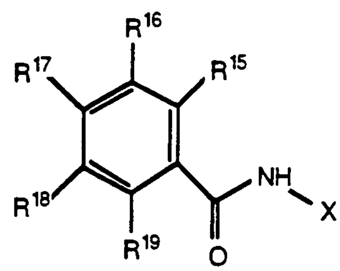
Novel compounds in accordance with the invention which are activated by amidase-like catalysis include compounds of the following formulas:

B. Prodrug Activation By Amidase Reaction

1. Aromatic or substituted aromatic amides, e.g., benzoate or substituted benzoate amides

Aromatic or substituted aromatic amide prodrug

Included in the invention is an aromatic amide B1a having the formula:

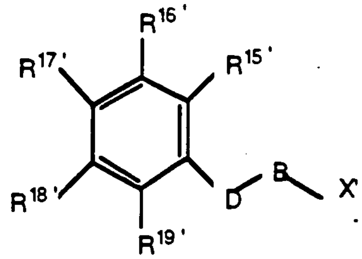


wherein X is a radical of the drug XNH_2. Advantageously, XNH_2 is a cytotoxic drug, such as doxorubicin or melphalan.

R^{15}, R^{16}, R^{17}, R^{18} and R^{19} are the same or different and are H, alkyl with 1-10 carbon atoms, alkoxy with 1-10 carbon atoms, monocyclic aromatic, alkene with 1-10 carbon atoms, hydroxy, hydroxyalkyl, aminoalkyl, thioalkyl, amino, alkylamino, alkylphosphonate, alkylsulfonate, alkylcarboxylate, or alkylammonium.

Hapten

Useful as a hapten as well as a prodrug is a compound B1b having the formula:



wherein X' is an analog of X of compound B1a, and X' is optionally linked to the carrier protein,

B is O, S, NH, or CH_2,

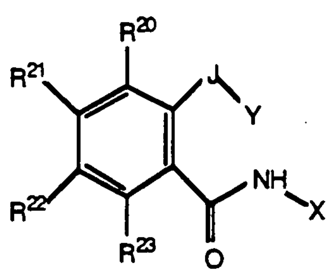
D is P(O)OH, SO_2, CHOH or SO with any stereochemistry, if D is CHOH then B is CH_2, and

$R^{15'}$, $R^{16'}$, $R^{17'}$, $R^{18'}$ and $R^{19'}$ are the same or different, are optionally linked to the carrier protein, and are H, alkyl with 1-10 carbon atoms, alkoxy with 1-10 carbon atoms, monocyclic aromatic, alkene with 1-10 carbon atoms, hydroxy, hydroxyalkyl, aminoalkyl, thioalkyl, amino, alkylamino, alkylphosphonate, alkylsulfonate, alkylcarboxylate, or alkylammonium.

2. Aromatic or substituted aromatic amides activated by an intramolecular nucleophilic attack on the amide carbonyl

Aromatic or substituted aromatic amide prodrug

Included in the invention is an aromatic amide compound B2a having the formula:



wherein X is a radical of the drug XNH_2. Advantageously, XNH_2 is a cytotoxic drug such as doxorubicin or melphalan.

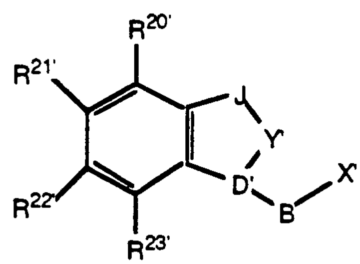
J is alkyl with 1-9 atoms in a linear configuration, alkyl with heteroatoms with 1-9 atoms in a linear configuration which have substituents that are phenyl, alkyl , or alkyl with heteroatoms,

Y is OH, NH_2, NHR or SH where R is an alkyl, alkenyl or alkynyl optionally substituted by one or more substituents selected from the group consisting of -OH, chloro, fluoro, bromo, iodo, -SO3, aryl, -SH, -(CO)H, -(CO)OH, ester groups, ether groups, -CO-, cyano, epoxide groups and heteroatoms, and

R^{20}, R^{21}, R^{22}, and R^{23} are the same or different and are H, alkyl with 1-10 carbon atoms, alkoxy with 1-10 carbon atoms, monocyclic aromatic, alkene with 1-10 carbon atoms, hydroxy, hydroxyalkyl, aminoalkyl, thioalkyl, amino, alkylamino, alkylphosphonate, alkylsulfonate, alkylcarboxylate, or alkylammonium.

Hapten

Useful as a hapten as well as a prodrug is a compound B2b having the formula:



wherein X' is an analog of the drug XNH_2 of compound B2a, and X' is optionally linked to a carrier protein,

B is O, S, NH, or CH_2,

D' is P(O), COH with any stereochemistry, if D' is COH then B and Y' are CH_2,

Y' is O, NH, NR, S or CH_2 where R is an alkyl, alkenyl or alkynyl optionally substituted by one or more substituents selected from the group consisting of -OH, chloro, fluoro, bromo, iodo, -SO3, aryl, -SH, -(CO)H, -(CO)OH, ester groups, ether groups, -CO-, cyano, epoxide groups and heteroatoms,

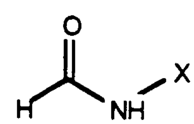
J is alkyl with 1-9 atoms in a linear configuration, alkyl with heteroatoms with 1-9 atoms in a linear configuration which have substituents that are phenyl, alkyl, or alkyl with heteroatoms, and;

$R^{20'}$, $R^{21'}$, $R^{22'}$, and $R^{23'}$ are the same or different, are optionally linked to the carrier protein and are H, alkyl with 1-10 carbon atoms, alkoxy with 1-10 carbon atoms, monocyclic aromatic, alkene with 1-10 carbon atoms, hydroxy, hydroxyalkyl, aminoalkyl, thioalkyl, amino, alkylamino, alkylphosphonate, alkylsulfonate, alkylcarboxylate, or alkylammonium.

3. Formylamides

Formylamide Prodrug

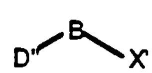
Included in the invention is a formylamide compound B3a having the formula:



wherein X is a radical of the drug XNH_2. Advantageously, XNH_2 is a cytotoxic drug such as doxorubicin or melphalan.

Hapten

Useful as a hapten as well as a prodrug is a compound B3b having the formula:



wherein X' is an analog of X of compound B3a, and X' is optionally linked to the carrier protein,

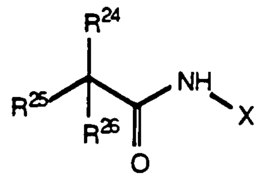
B is O, S, NH, or CH_2, and

D'' is HP(O)OH, CH_2OH, $P(O)(OH)_2$, or SO_3H, if D'' is CH_2OH then B is CH_2.

4. Acetylamides

Acetylamide Prodrug

Included in the invention is an acetylamide compound B4a having the formula:

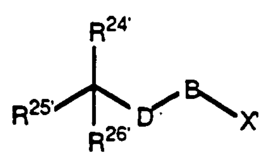


wherein X is a radical of the drug XNH_2. Advantageously, XNH_2 is a cytotoxic drug such as doxorubicin or melphalan.

R^{24}, R^{25} and R^{26} are the same or different and are H, alkyl with 2 to 22 carbon atoms, alkyl with heteroatoms, cycloalkyldiol, monocyclic aromatic, alkylphosphonate, alkylsulfonate, alkylcarboxylate, alkylammonium or alkene.

Hapten

Useful as a hapten as well as a prodrug is a compound B4b having the formula:



wherein X' is an analog of X of compound B4a, and X' is optionally linked to the carrier protein,

B is O, S, NH, or CH_2,

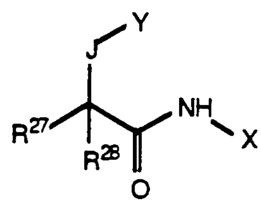
D is P(O)OH, SO_2, CHOH or SO with any stereochemistry, if D is CHOH then B is CH_2, and

$R^{24'-26'}$ which are optionally linked to a carrier protein, are the same or different and are H, alkyl with 2 to 22 carbon atoms, alkyl with heteroatoms, cycloalkyldiol, monocyclic aromatic, alkylphosphonate, alkylsulfonate, alkylcarboxylate, alkylammonium or alkene.

5. Acetylamides activated by an intramolecular nucleophilic attack on the amide carbonyl

Acetylamide Prodrug

Included in the invention is an acetylamide compound B5a having the formula:



wherein X is a radical of the drug XNH_2. Advantageously, XNH_2 is a cytotoxic drug such as doxorubicin or melphalan.

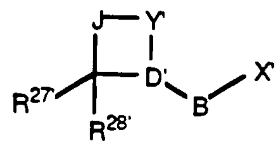
J is alkyl with 1-9 atoms in a linear configuration, alkyl with heteroatoms with 1-9 atoms in a linear configuration which have substituents that are phenyl, alkyl, or alkyl with heteroatoms,

Y is OH, NH2, NHR or SH where R is an alkyl. alkenyl or alkynyl optionally substituted by one or more substituents selected from the group consisting of -OH, chloro, fluoro, bromo, iodo, -SO3, aryl, -SH, -(CO)H, -(CO)OH, ester groups, ether groups, -CO-, cyano, epoxide groups and heteroatoms, and

R^{27-28} are the same or different and are H, alkyl with 2 to 22 carbon atoms, alkyl with heteroatoms, cycloalkyldiol, monocyclic aromatic, alkylphosphonate, alkylsulfonate, alkylcarboxylate, alkylammonium or alkene.

Hapten

Useful as a hapten as well as a prodrug is a compound B5b having the formula:



wherein X' is an analog of X of compound B5a, and X' is optionally linked to the carrier protein,

B is O, S, NH, or CH_2,

D' is P(O), COH with any stereochemistry, if D' is COH then B and Y' are CH_2.

Y' is O, NH, NR, S or CH_2 where R is an alkyl, alkenyl or alkynyl optionally substituted by one or more substituents selected from the group consisting of -OH, chloro, fluoro, bromo, iodo, -SO3, aryl, -SH, -(CO)H, -(CO)OH, ester groups, ether groups, -CO-, cyano, epoxide groups and heteroatoms,

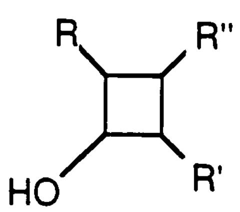
J is alkyl with 1-9 atoms in a linear configuration, alkyl with heteroatoms with 1-9 atoms in a linear configuration which have substituents that are phenyl, alkyl, or alkyl with heteroatoms, and

$R^{27'-28'}$ which are optionally linked to the carrier protein are the same or different and are H, alkyl with 2 to 22 carbon atoms, alkyl with heteroatoms, cycloalkyldiol, monocyclic aromatic, alkylphosphonate, alkylsulfonate, alkylcarboxylate, alkylammonium or alkene.

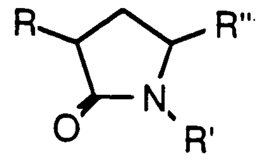
6. Monobactam hydrolysis.

Overview Of Hapten Strategies For Raising β-Lactamase Antibodies

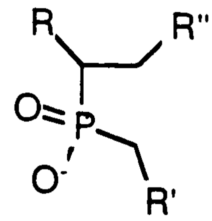
It is necessary to design strategies and prepare haptens for immunization to elicit antibodies capable of monocyclic β-lactam ("monobactam") hydrolysis. Some possibilities of design are shown below.



1



2



3

The strategy of Compound 1 which differs from the β-lactam substrate in that the β-lactam ring has been replaced with cyclobutanol (such that a secondary alcohol replaces the β-lactam carbonyl). Alcohol transition state analogs have been successfully designed and are well known as transition state inhibitors in enzymology (Bolis, G., et al., J. Med. Chem. 30(1987):1729-37) and have been used to raise hydrolytic catalytic antibodies (Shokat, K. M., et al., Chem. Int. Ed. Engl. 29 (1990):1296-1303).

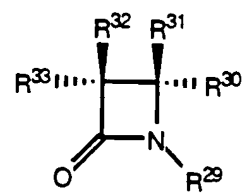
The strategy of Compound 2 involves the addition of a methylene group to the β-lactam ring to form a γ-lactam ring. Because of the difference in ring size (four- versus five-membered), the bond angle of the carbonyls will differ with respect to their respective rings. The carbonyl of the γ-lactam will be more out of plane of the ring (more tetrahedral) than the β-lactam carbonyl (Baldwin, J. E., et al., Tetrahedron 42 (1986):4879). This difference will cause substrate destabilization of the β-lactam to a γ-lactam-elicited antibody, contributing to catalysis.

Non-cyclic hapten 3 utilizes a combination of substrate destabilization and transition state complementarity to induce an antibody with β-lactamase activity. This or similar compounds will be linear analogs of the β-lactam in which the scissile bond has been replaced by the transition state-like dialkylphosphinate (shown here), or similar phosphorous-based group. Thus in this strategy, there is a combination of transition state analogy and ground state destabilization.

In all strategies, the structure of the substituents will depend on the drug (occupying R") conjugation to an immunogenic carrier protein including but not limited to KLH or BSA (through R, R', or R") and the structure of the antibiotic (R and R") used in screening mutants.

Monolactam Prodrug

Included in the invention is a monolactam compound B6a having the formula:



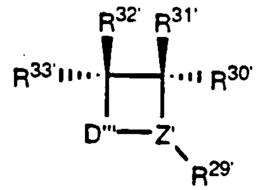
wherein at least one of R^{30} and R^{31} is OX where X is a radical of the drug XOH. Advantageously, XOH is a cytotoxic drug such as an antineoplastic nucleoside analog (joined to the β-lactam moiety at the 3' and/or 5' oxygen of the aldose ring), doxorubicin, or the enol form of aldophosphamide.

R^{29-33} which are not OX are the same or different and are H, alkyl with 1-10 carbon atoms, alkenyl with 1-10 carbon atoms, monocyclic aromatic, carboxyalkyl with 1-10 carbon atoms and with or without heterocyclic or phenyl substitution (optionally substituted on the heterocyclic or phenyl group), alkoxy with 1-10 carbon atoms, alkylamino with 1-10 carbon atoms, aminoalkyl with 1-10 carbon atoms, acyloxy with 1-10 carbon atoms, with or without heterocyclic or phenyl substitution (optionally substituted on the heterocyclic or phenyl group), or acylamino with 1-10 carbon atoms with or without heterocyclic or phenyl substitution (optionally substituted on the heterocyclic or phenyl group), and

R^{29} is optionally SO_3H or SO_4H.

Hapten

Useful as a hapten as well as a prodrug is a compound B6b having the formula:



wherein at least one of $R^{30'}$ and $R^{31'}$ is an analog of X of compound B6a, and said analog is optionally linked to a carrier protein,

D''' is SO_2, SO or CHOH with any stereochemistry, if D''' is CHOH then Z' is CH,

Z' is O, N, or CH with any stereochemistry; when Z' is O then $R^{29'}$ is omitted,

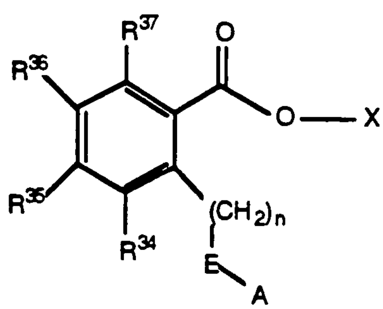
$R^{29'-33'}$ which are not said analog, are the same or different and are H, alkyl with 1-10 carbon atoms, alkenyl with 1-10 carbon atoms, monocyclic aromatic, carboxyalkyl with 1-10 carbon atoms and with or without heterocyclic or phenyl substitution (optionally substituted on the heterocyclic or phenyl group), alkoxy with 1-10 carbon atoms, alkylamino with 1-10 carbon atoms, aminoalkyl with 1-10 carbon atoms, acyloxy with 1-10 carbon atoms, with or without heterocyclic or phenyl substitution (optionally substituted on the heterocyclic or phenyl group), or acylamino with 1-10 carbon atoms with or without heterocyclic or phenyl substitution (optionally substituted on the heterocyclic or phenyl group), and

$R^{29'}$ is optionally SO_3H or SO_4H, and

$R^{29'-33'}$ are optionally linked to a carrier protein.

Monolactam Prodrug

Included in the invention is a monolactam compound B6c having the formula:



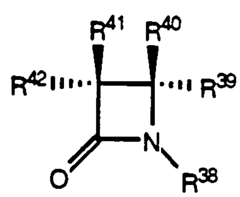
wherein X is a radical of a drug XOH. Advantageously, XOH is a cytotoxic drug such as an antineoplastic nucleoside analog (joined to the carboxyl moiety at the 3' and/or 5' position of the aldose ring), doxorubicin or the enol form of aldophosphamide.

R^{34}, R^{35}, R^{36}, and R^{37} are the same or different and are H, alkyl with 1-10 carbon atoms, alkoxy with 1-10 carbon atoms, monocyclic aromatic, alkene with 1-10 carbon atoms, hydroxy, hydroxyalkyl, aminoalkyl, thioalkyl, amino, alkylamino, alkylphosphonate, alkylsulfonate, alkylcarboxylate, or alkylammonium,

n is an integer from O to 3,

E is optionally present and is oxygen, carbonyloxy, or oxycarbonyl,

A is the radical:



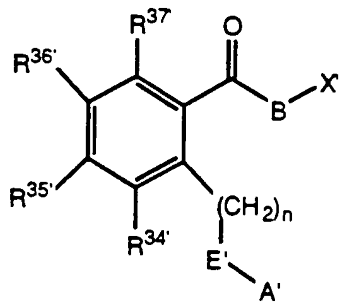
and R^{38}, R^{39}, R^{40}, R^{41} or R^{42} is the site of attachment to E, or if E is not present to $[CH_2]n$ or if E is not present and n = o, to the phenyl ring.

R^{38}, R^{39}, R^{40}, R^{41} and R^{42} are the same or different and are H, alkyl with 1-10 carbon atoms, alkenyl with 1-10 carbon atoms, monocyclic aromatic, carboxyalkyl with 1-10 carbon atoms and with or without heterocyclic or phenyl substitution (optionally substituted on the heterocyclic or phenyl group), alkoxy with 1-10 carbon atoms, alkylamino with 1-10 carbon atoms, aminoalkyl with 1-10 carbon atoms, acyloxy with 1-10 carbon atoms, with or without heterocyclic or phenyl substitution (optionally substituted on the heterocyclic or phenyl group), or acylamino with 1-10 carbon atoms with or without heterocyclic or phenyl substitution (optionally substituted on the heterocyclic or phenyl group), and

R^{38} is optionally SO_3H or SO_4H.

Hapten

Useful as a hapten as well as a prodrug is a compound B6d having the formula:



wherein X' is an analog of X of compound B6c, and is optionally linked to carrier protein,

B is O, S, NH, or CH_2,

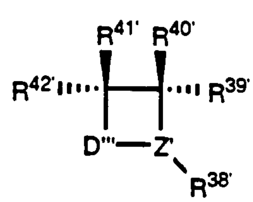
$R^{34'}$, $R^{35'}$, $R^{36'}$, and $R^{37'}$ are the same or different and are H, alkyl with 1-10 carbon atoms, alkoxy with 1-10 carbon atoms, monocyclic aromatic, alkene with 1-10 carbon atoms, hydroxy, hydroxyalkyl, aminoalkyl, thioalkyl, amino, alkylamino, alkylphosphonate, alkylsulfonate, alkylcarboxylate, or alkylammonium, with the proviso that at least 1 of $R^{34'-37'}$ is not H,

$R^{34'}$, $R^{35'}$, $R^{36'}$ or $R^{37'}$ is optionally the site of attachment to a carrier protein,

n is an integer from 0 to 3,

E' is optionally present and is CH_2, O, carbonyloxy, carbonyl methylene, oxycarbonyl, or methylenecarbonyl,

A' is the radical:



wherein D''' is SO_2, SO or CHOH with any stereochemistry, if D''' is CHOH, Z' is CH,

Z' is O, N, or CH with any stereochemistry; when Z' is O then $R^{38'}$ is omitted,

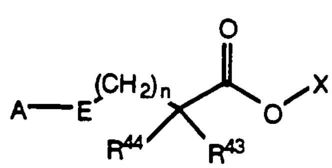
and $R^{38'}$, $R^{39'}$, $R^{40'}$, $R^{41'}$ or $R^{42'}$ is the site of attachment to E', or if E' is not present to $(CH_2)n$, or if E is not present and n = o, to the phenyl ring.

$R^{38'}$, $R^{39'}$, $R^{40'}$, $R^{41'}$ and $R^{42'}$ are the same or different and are H, alkyl with 1-10 carbon atoms, alkenyl with 1-10 carbon atoms, monocyclic aromatic, carboxyalkyl with 1-10 carbon atoms and with or without heterocyclic or phenyl substitution (optionally substituted on the heterocyclic or phenyl group), alkoxy with 1-10 carbon atoms, alkylamino with1-10 carbon atoms, aminoalkyl with 1-10 carbon atoms, acyloxy with 1-10 carbon atoms, with or without heterocyclic or phenyl substitution (optionally substituted on the heterocyclic or phenyl group), or acylamino with 1-10 carbon atoms with or without heterocyclic or phenyl substitution (optionally substituted on the heterocyclic or phenyl group), and

$R^{38'}$ is optionally SO_3H or SO_4H.

Monolactam Di or Tri-substituted Acetate Prodrug

Included in the invention is a monolactam compound B6e having the formula:



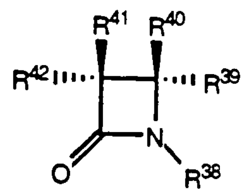
X is a radical of the drug XOH. Advantageously, XOH is a cytotoxic drug such as an antineoplastic nucleoside analog (joined to the carboxyl moiety at the 3' and/or 5' position of the aldose ring), doxorubicin, or the enol form of aldophosphamide.

n is an integer from 0 to 4,

R^{43} and R^{44} are the same or different but both are not H, and are alkyl with 2 to 22 carbon atoms, alkyl with heteroatoms, cycloalkyldiol, monocyclic aromatic, alkylphosphonate, alkylsulfonate, alkylcarboxylate, alkylammonium or alkene,

E is optionally present and is oxygen, carbonyloxy, or oxycarbonyl,

A is the following radical:



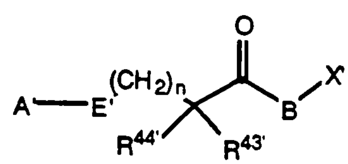
wherein R^{38}, R^{39}, R^{40}, R^{41} or R^{42} is the site of attachment to E, or if E is not present to (CH2)n, or if E is not present and n = o, t the carbon atom to which R^{43} and R^{44} are attached.

R^{38}, R^{39}, R^{40}, R^{41} and R^{42} are the same or different and are H, alkyl with 1-10 carbon atoms, alkenyl with 1-10 carbon atoms, monocyclic aromatic, carboxyalkyl with 1-10 carbon atoms and with or without heterocyclic or phenyl substitution (optionally substituted on the heterocyclic or phenyl group), alkoxy with 1-10 carbon atoms, alkylamino with 1-10 carbon atoms, aminoalkyl with 1-10 carbon atoms, acyloxy with 1-10 carbon atoms, with or without heterocyclic or phenyl substitution (optionally substituted on the heterocyclic or phenyl group), or acylamino with 1-10 carbon atoms with or without heterocyclic or phenyl substitution (optionally substituted on the heterocyclic or phenyl group), and

R^{38} is optionally SO_3H or SO_4H.

Hapten

Useful as a hapten as well as a prodrug is a compound B6f having the formula:



wherein X' is an analog of X of compound B6e, and X' is optionally linked to carrier protein,

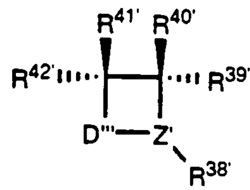
B is O, S, NH, or CH_2,

n is an interger from 0 to 4,

$R^{43'}$ and $R^{44'}$ are the same or different but both are not H, and are alkyl with 2 to 22 carbon atoms, alkyl with heteroatoms, cycloalkyldiol, monocyclic aromatic, alkylphosphonate, alkylsulfonate, alkylcarboxylate, alkylammonium or alkene,

E' is optionally present and is CH_2, O, carbonyloxy, carbonyl methylene, oxycarbonyl, or methylenecarbonyl,

A' is the radical:



wherein D''' is SO_2, SO or CHOH with any stereochemistry, if D''' is CHOH, Z' is CH,

Z' is O, N, or CH with any stereochemistry, when Z' is O then $R^{38'}$ is omitted,

$R^{38'}$, $R^{39'}$, $R^{40'}$, $R^{41'}$ or $R^{42'}$ is the site of attachment to to E', or if E' is not present to $(CH_2)n$, or if E' is not present and n = o, to the carbon atom to which $R^{34'}$ and $R^{44'}$ are attached,

$R^{38'}$, $R^{39'}$, $R^{40'}$, $R^{41'}$ and $R^{42'}$ are the same or different and are H, alkyl with 1-10 carbon atoms, alkenyl with 1-10 carbon atoms, monocyclic aromatic, carboxyalkyl with 1-10 carbon atoms and with or without heterocyclic or phenyl substitution (optionally substituted on the heterocyclic or phenyl group), alkoxy with 1-10 carbon atoms, alkylamino with 1-10 carbon atoms, aminoalkyl with 1-10 carbon atoms, acyloxy with 1-10 carbon atoms, with or without heterocyclic or phenyl substitution (optionally substituted on the heterocyclic or phenyl group), or acylamino with 1-10 carbon atoms with or without heterocyclic or phenyl substitution (optionally substituted on the heterocyclic or phenyl group), and

$R^{38'}$ is optionally SO_3H or SO_4H.

Acetal Hydrolase Catalysis

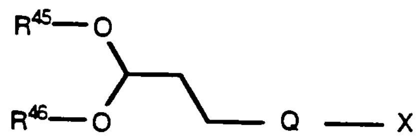
Novel compounds in accordance with the invention which are activated by acetal hydrolase or ortho-ester hydrolase catalysis include compounds of the following formulas:

C. <u>Prodrug Activation By Acetal Hydrolysis Reaction</u>

1. Dialkyl acetals

Dialkyl acetal prodrug

Included in the invention is an alkyl acetal compound C1a having the formula:



wherein X is a radical of the drug XQH. Advantageously XQH is a cytotoxic drug such as a nucleoside analog or phosphoramide mustard [$HOP(O)(NH_2)N(CH_2CH_2Cl)_2$], melphalan or doxorubicin.

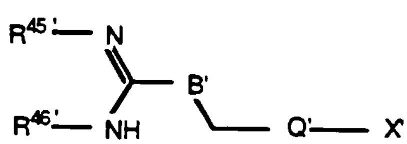
where Q is O or NH, and

R^{45} and R^{46} are the same or different and are alkyl unsubstituted, alkyl substituted with halogens, heteroatoms, phosphonate, sulfonate, carboxylate, alkylammonium, alkene, or monocyclic aromatic.

The compound is not Aldophosphamide diethylacetal. However, Aldophosphamide diethylacetal is useful in the methods of treatment utilizing catalytic antibodies of the subject invention.

Hapten 1

Useful as a hapten as well as a prodrug is a compound C1b having the formula:



wherein Q' is O, S, NH, or CH_2,

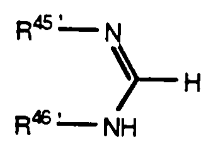
X' is an analog of X of compound C1a, and X' is optionally linked to a carrier protein,

B' is NH or CH_2, if B' is NH, then Q' is CH_2, and

$R^{45'}$ and $R^{46'}$ are the same or different and are H, alkyl unsubstituted, alkyl substituted with halogens, heteroatoms, phosphonate, sulfonate, carboxylate, alkylammonium, alkene, or monocyclic aromatic, and are optionally linked to a carrier protein.

Hapten 2

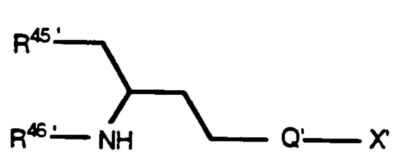
Useful as a hapten as well as a prodrug is a compound C1c having the formula:



wherein $R^{45'}$ and $R^{46'}$ are the same or different and are H, alkyl unsubstituted, alkyl substituted with halogens, heteroatoms, phosphonate, sulfonate, carboxylate, alkylammonium, alkene, or monocyclic aromatic, and are optionally linked to a carrier protein.

Hapten 3

Useful as a hapten as well as a prodrug is a compound C1d having the formula:



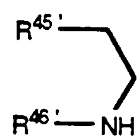
wherein Q' is O, S, NH, or CH2,

wherein X' is an analog of X of compound C1a, and X' is optionally linked to a carrier protein, and

$R^{45'}$ and $R^{46'}$ are the same or different and are H, alkyl unsubstituted, alkyl substituted with halogens, heteroatoms, phosphonate, sulfonate, carboxylate, alkylammonium, alkene, or monocyclic aromatic, and are optionally linked to a carrier protein.

Hapten 4

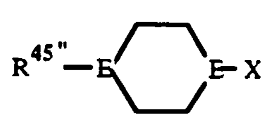
Useful as a hapten as well as a prodrug is a compound C1e having the formula:



wherein $R^{45'}$ and $R^{46'}$ are the same or different and are H, alkyl unsubstituted, alkyl substituted with halogens, heteroatoms, phosphonate, sulfonate, carboxylate, alkylammonium, alkene, or monocyclic aromatic, and are optionally linked to a carrier protein.

Hapten 5

Useful as a hapten as well as a prodrug is a compound C1F having the formula:



wherein X' is an analog of X of compound C1a;

wherein E and E' are the same or different and are N, C, O or S.

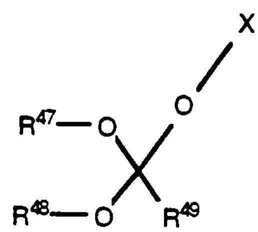
wherein $R^{45''}$ is H, aminocarboxy, alkyl unsubstituted, alkyl substituted with halogens, heteroatoms, phosphonate, sulfonate, carboxylate, alkylammonium, alkene or monocyclic aromatic, and are optionally linked to a carrier protein.

When E which is linked to $R^{45''}$, is N, it is preferred that the E-$R^{45''}$, linkage forms an amide substituted with an amine moiety.

2. Ortho esters

Ortho ester prodrug

Included in the invention is an orthoester compound C2a having the formula:



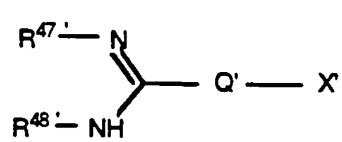
wherein X is a radical of the drug XOH. Advantageously, XOH is a cytotoxic drug such as a nucleoside analog or doxorubicin or the enol form of aldophosphamide.

R^{47}, R^{48}, and R^{49} are the same or different and are alkyl unsubstituted, alkyl substituted with halogens, heteroatoms, phosphonate, sulfonate, carboxylate, alkylammonium, alkene, or monocyclic aromatic, and

R^{49} is optionally H.

Hapten 1

Useful as a hapten as well as a prodrug is a compound C2b having the formula:



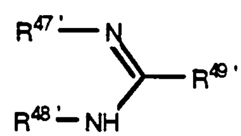
wherein X' is an analog of X of compound C2a, and which is optionally linked to a carrier protein,

Q' is O, CH_2, S, or NH, and

$R^{47'}$ and $R^{48'}$ are the same or different and are H, alkyl unsubstituted, alkyl substituted with halogens, heteroatoms, phosphonate, sulfonate, carboxylate, alkylammonium, alkene, or monocyclic aromatic, and are optionally linked to a carrier protein.

Hapten 2

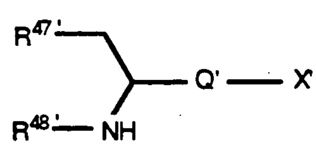
Useful as a hapten as well as a prodrug is a compound C2c having the formula:



wherein $R^{47'}$, $R^{48'}$, and $R^{49'}$ are the same or different and are H, alkyl unsubstituted, alkyl substituted with halogens, heteroatoms, phosphonate, sulfonate, carboxylate, alkylammonium, alkene, or monocyclic aromatic, and are optionally linked to a carrier protein.

Hapten 3

Useful as a hapten as well as a prodrug is a compound C2d having the formula:



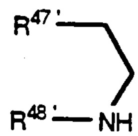
wherein X' is an analog of X of compound C2a, and which is optionally linked to a carrier protein,

Q' is CH_2, and

$R^{47'}$ and $R^{48'}$ are the same or different and are H, alkyl unsubstituted, alkyl substituted with halogens, heteroatoms, phosphonate, sulfonate, carboxylate, alkylammonium, alkene, or monocyclic aromatic, and are optionally linked to a carrier protein.

Hapten 4

Useful as a hapten as well as a prodrug is a compound C2e having the formula:

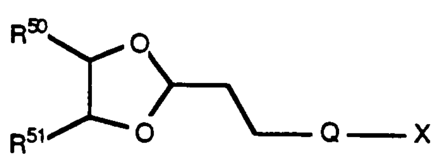


wherein $R^{47'}$ and $R^{48'}$ are the same or different and are H, alkyl unsubstituted, alkyl substituted with halogens, heteroatoms, phosphonate, sulfonate, carboxylate, alkylammonium, alkene, or monocyclic aromatic, and are optionally linked to a carrier protein.

3. Diol acetals, e.g., sugar-substituted acetals

Diol acetal prodrug

Included in the invention is a diol acetal compound C3a having the formula:



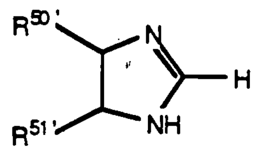
wherein X is a radical of the drug XQH. Advantageously, XQH is a cytotoxic drug such as a nucleoside analog or phosphoramide mustard [$HOP(O)(NH_2)N(CH_2CH_2Cl)_2$], melphalan or doxorubicin.

Q is O or NH, and

R^{50} and R^{51} are the same or different and are H, alkyl unsubstituted, alkyl substituted with halogens, heteroatoms, phosphonate, sulfonate, carboxylate or alkyl ester or alkyl amide, hydroxyl, alkylammonium, amino, alkene, or monocyclic aromatic. Advantageously, R^{50} and R^{51} are cis and the same so that there is a mirror plane of symmetry within the acetal moiety of the molecule, and the number of isomers is minimized.

Hapten 1

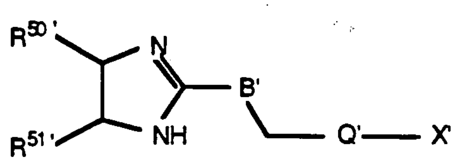
Useful as a hapten as well as a prodrug is a compound C3b having the formula:



wherein $R^{50'}$ and $R^{51'}$ are the same or different and are H, alkyl unsubstituted, alkyl substituted with halogens, heteroatoms, phosphonate, sulfonate, carboxylate or alkyl ester or alkyl amide, hydroxyl, alkylammonium, amino, alkene, or monocyclic aromatic, and are optionally linked to a carrier protein.

Hapten 2

Useful as a hapten as well as a prodrug is a compound C3c having the formula:



wherein Q' is O, S, NH or CH_2,

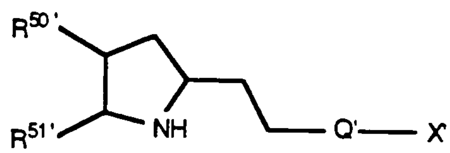
X' is an analog of X of compound C3a, and X' is optionally linked to a carrier protein,

B' is NH or CH_2, if B' is NH, then Q' is CH_2, and

$R^{50'}$ and $R^{51'}$ are the same or different and are H, alkyl unsubstituted, alkyl substituted with halogens, heteroatoms, phosphonate, sulfonate, carboxylate or alkyl ester or alkyl amide, hydroxyl, alkylammonium, amino, alkene, or monocyclic aromatic, and are optionally linked to a carrier protein.

Hapten 3

Useful as a hapten as well as a prodrug is a compound C3d having the formula:



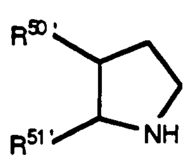
wherein Q' is O, S, NH or CH_2,

X' is an analog of X of compound C3a, and X' is optionally linked to a carrier protein, and

$R^{50'}$ and $R^{51'}$ are the same or different and are H, alkyl unsubstituted, alkyl substituted with halogens, heteroatoms, phosphonate, sulfonate, carboxylate or alkyl ester or alkyl amide, hydroxyl, alkylammonium, amino, alkene, or monocyclic aromatic, and are optionally linked to a carrier protein.

Hapten 4

Useful as a hapten as well as a prodrug is a compound C3e having the formula:

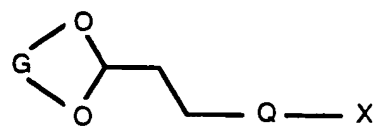


wherein $R^{50'}$ and $R^{51'}$ are the same or different and are H, alkyl unsubstituted, alkyl substituted with halogens, heteroatoms, phosphonate, sulfonate, carboxylate or alkyl ester or

alkyl amide, hydroxyl, alkylammonium, amino, alkene, or monocyclic aromatic, and are optionally linked to a carrier protein.

Sugar acetal prodrug

Included in the invention is a diol acetal compound C3f having the formula:

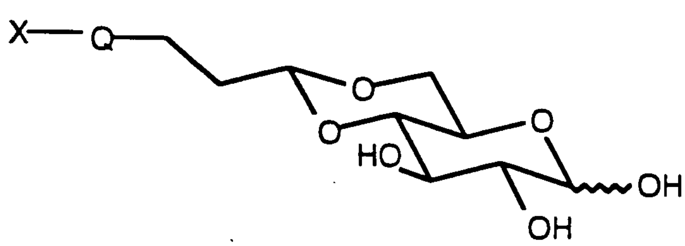


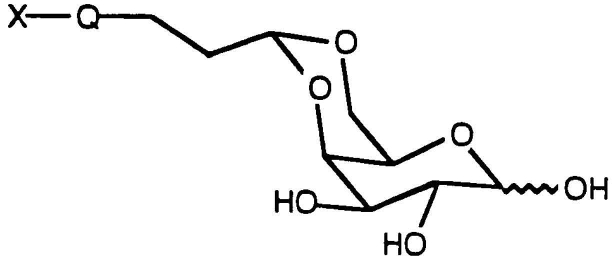
wherein X is a radical of a drug XQH. Advantageously, XQH is a cytotoxic drug such as a nucleoside analog or phosphoramide mustard [$HOP(O)(NH_2)N(CH_2CH_2Cl)_2$], melphalan or doxorubicin.

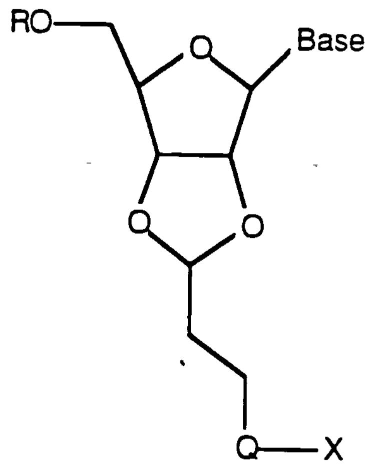
Q is O or NH, and

G is a radical of the diol $G(OH)_2$, $G(OH)_2$ is a sugar, cycloalkyldiol or ortho-phenyldiol, and G is optionally substituted with halogens, heteroatoms, phosphonate, sulfonate, carboxylate, alkylammonium, alkene, or monocyclic aromatic.

Examples of the above are as follows:





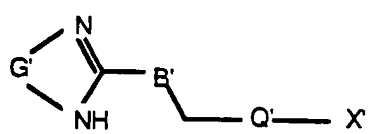


Base = Uracil, 5-Fluorouracil, Cytosine, Adenine, Guanine

R = H, PO_3H_2

Sugar acetal hapten 1

Useful as a hapten as well as a prodrug is a compound C3g having the formula:



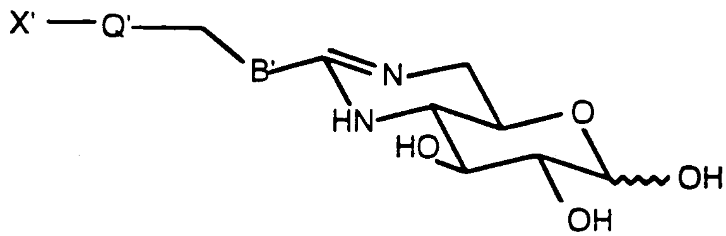
wherein Q' is O, S, NH or CH_2,

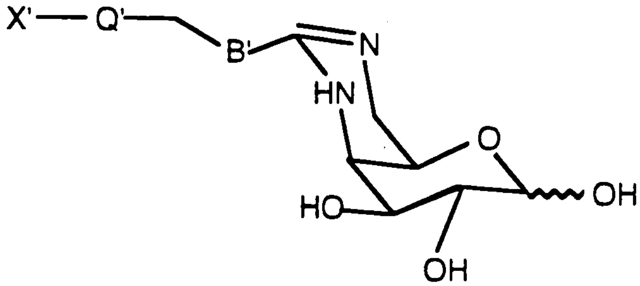
X' is an analog of X of compound C3f, and X' is optionally linked to a carrier protein,

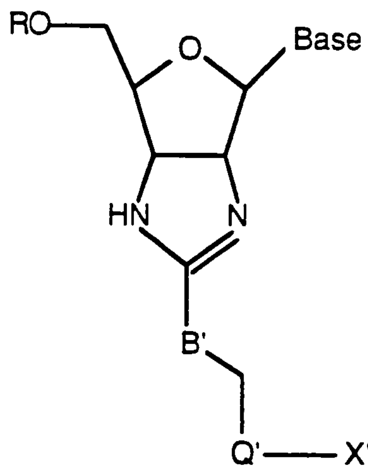
B' is NH or CH_2, if B' is NH, then Q' is CH_2, and

G' is a radical of the diol $G(OH)_2$, $G(OH)_2$ is a sugar, cycloalkyldiol or ortho-phenyldiol, and G' is optionally substituted with halogens, heteroatoms, phosphonate, sulfonate, carboxylate, alkylammonium, alkene, or monocyclic aromatic, and is optionally linked to a carrier protein.

Examples of the above are as follows:





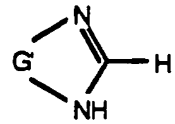


Base = Uracil, 5-Fluorouracil, Cytosine, Adenine, Guanine

R = H, PO_3H_2

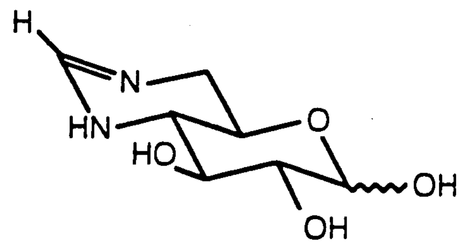
Sugar acetal hapten 2 .

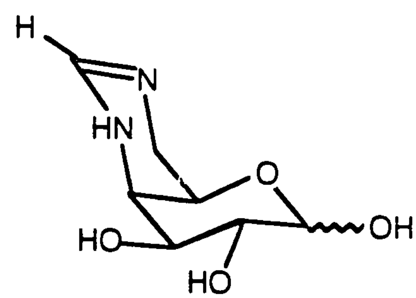
Useful as a hapten as well as a prodrug is a compound C3h having the formula:

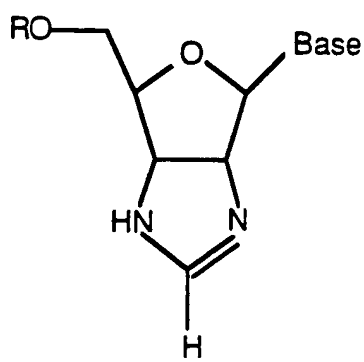


G' is a radical of the diol $G(OH)_2$, $G(OH)_2$ is a sugar, cycloalkyldiol or ortho-phenyldiol, and G' is optionally substituted with halogens, heteroatoms, phosphonate, sulfonate, carboxylate, alkylammonium, alkene, or monocyclic aromatic, and is optionally linked to a carrier protein.

Examples of the above are as follows:





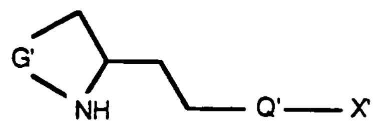


Base = Uracil, 5-Fluorouracil, Cytosine, Adenine, Guanine or analogues thereof

R = H, PO_3H_2

Sugar acetal hapten 3

Useful as a hapten as well as a prodrug is a compound C3i having the formula:

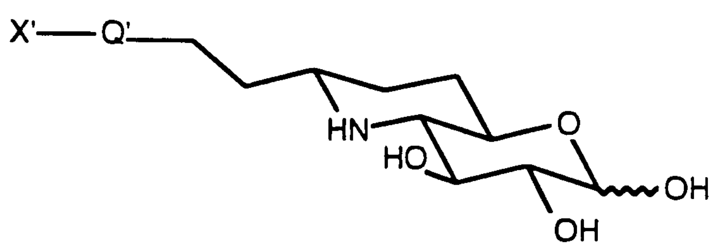


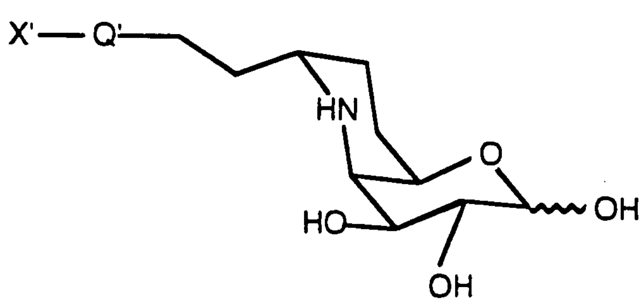
wherein Q' is O, S, NH or CH_2,

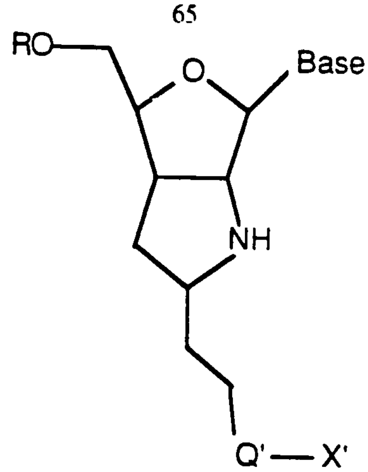
X' is an analog of X of compound C3f, which is optionally linked to a carrier protein, and

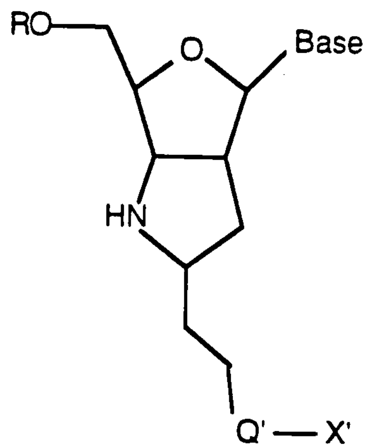
G' is a radical of the diol $G(OH)_2$, $G(OH)_2$ is a sugar, cycloalkyldiol or ortho-phenyldiol, and G' is optionally substituted with halogens, heteroatoms, phosphonate, sulfonate, carboxylate, alkylammonium, alkene, or monocyclic aromatic, and are optionally linked to a carrier protein.

Examples of the above are as follows:







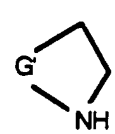


Base = Uracil, 5-Fluorouracil, Cytosine, Adenine, Guanine or analogues thereof

R = H, PO_3H_2

Sugar acetal hapten 4

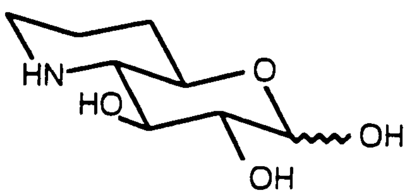
Useful as a hapten as well as a prodrug is a compound C3j having the formula:

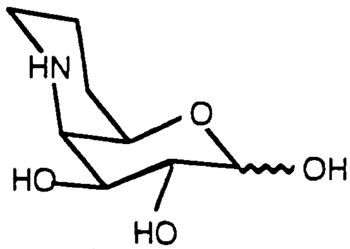


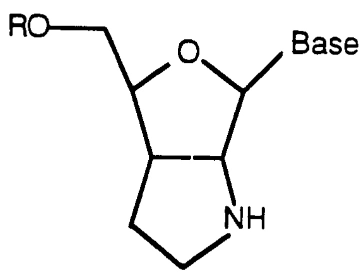
G' is a radical of the diol $G(OH)_2$, $G(OH)_2$ is a sugar, cycloalkyldiol or ortho-phenyldiol, and G' is optionally substituted with halogens, heteroatoms, phosphonate,

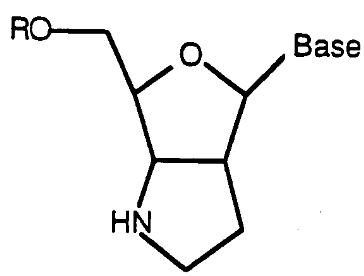
sulfonate, carboxylate, alkylammonium, alkene, or monocyclic aromatic, and are optionally linked to a carrier protein.

Examples of the above are as follows:









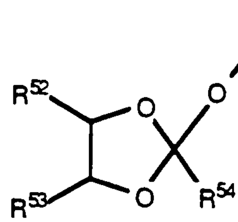
Base = Uracil, 5-Fluorouracil, Cytosine, Adenine, Guanine or analogues thereof

R = H, PO_3H_2

4. Diol ortho esters

Diol orthoester prodrug

Included in the invention is a diol orthoester compound C4a having the formula:

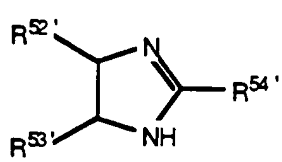


wherein X is a radical of a drug XOH. Advantageously, XOH is a cytotoxic drug such as a nucleoside analog or doxorubicin or the enol form of aldophosphamide.

R^{52}, R^{53} and R^{54} are the same or different and are H, alkyl unsubstituted, alkyl substituted with halogens, heteroatoms, phosphonate, sulfonate, carboxylate or alkyl ester or alkyl amide, hydroxyl, alkylammonium, amino, alkene, or monocyclic aromatic. Advantageously, R^{52} and R^{53} are cis and the same so that there is a mirror plane of symmetry within the cyclic acetal moiety of the molecule, and the number of isomers is minimized.

Diol orthoester hapten 1

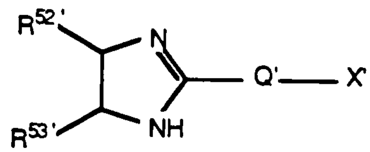
Useful as a hapten as well as a prodrug is a compound C4b having the formula:



wherein $R^{52'}$, $R^{53'}$, and $R^{54'}$ are the same or different, and are H, alkyl unsubstituted, alkyl substituted with halogens, heteroatoms, phosphonate, sulfonate, carboxylate or alkyl ester or alkyl amide, hydroxyl, alkylammonium, amino, alkene, or monocyclic aromatic, and are optionally linked to a carrier protein.

Diol orthoester hapten 2

Useful as a hapten as well as a prodrug is a compound C4c having the formula:



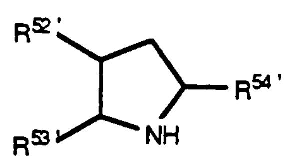
wherein X' is an analog of X of compound C4a, and which is optionally linked to a carrier protein,

Q' is CH_2 or NH, and

$R^{52'}$ and $R^{53'}$ are the same or different, and are H, alkyl unsubstituted, alkyl substituted with halogens, heteroatoms, phosphonate, sulfonate, carboxylate or alkyl ester or alkyl amide, hydroxyl, alkylammonium, amino, alkene, or monocyclic aromatic, and are optionally linked to a carrier protein.

Diol orthoester hapten 3

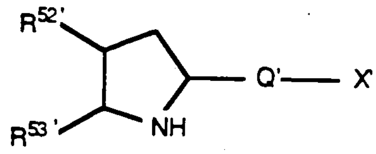
Useful as a hapten as well as a prodrug is a compound C4d having the formula:



wherein $R^{52'}$, $R^{53'}$, and $R^{54'}$ are the same or different, and are H, alkyl unsubstituted, alkyl substituted with halogens, heteroatoms, phosphonate, sulfonate, carboxylate or alkyl ester or alkyl amide, hydroxyl, alkylammonium, amino, alkene, or monocyclic aromatic, and are optionally linked to a carrier protein.

Diol orthoester hapten 4

Useful as a hapten as well as a prodrug is a compound C4e having the formula:



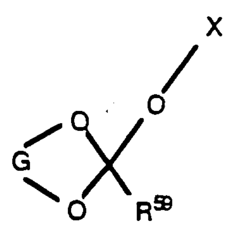
wherein X' is an analog of X of compound C4a, and which is optionally linked to a carrier protein,

Q' is CH_2, and

$R^{52'}$ and $R^{53'}$ are the same or different, and are H, alkyl unsubstituted, alkyl substituted with halogens, heteroatoms, phosphonate, sulfonate, carboxylate or alkyl ester or alkyl amide, hydroxyl, alkylammonium, amino, alkene, or monocyclic aromatic, and are optionally linked to a carrier protein.

Sugar orthoester prodrug

Included in the invention is a diol orthoester compound C4f having the formula:

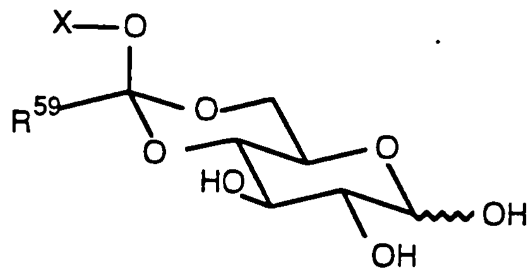


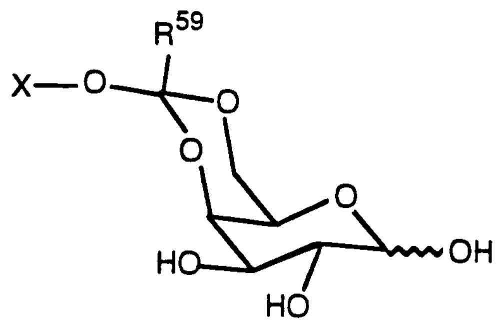
wherein X is a radical of a drug XOH. Advantageously, XOH is a cytotoxic drug such as a nucleoside analog, the enol form of aldophosphamide or doxorubicin.

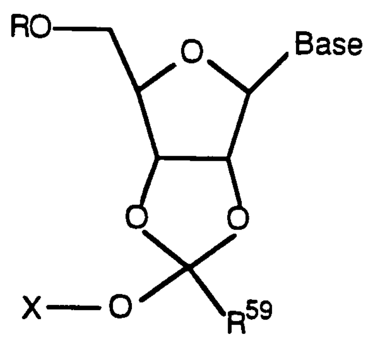
G is a radical of the diol $G(OH)_2$, $G(OH)_2$ is a sugar, cycloalkyldiol or ortho-phenyldiol, and G is optionally substituted with halogens, heteroatoms, phosphonate, sulfonate, carboxylate, alkylammonium, alkene, or monocyclic aromatic, and

R^{59} is H, alkyl unsubstituted, alkyl substituted with halogens, heteroatoms, phosphonate, sulfonate, carboxylate or alkyl ester or alkyl amide, hydroxyl, alkylammonium, amino, alkene, or monocyclic aromatic.

Examples of the above are as follows:





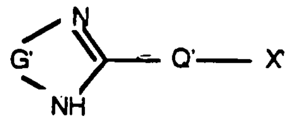


Base = Uracil, 5-Fluorouracil, Cytosine, Adenine, Guanine or analogues thereof

R = H, PO_3H_2

Sugar orthoester hapten 1

Useful as a hapten as well as a prodrug is a compound C4g having the formula:

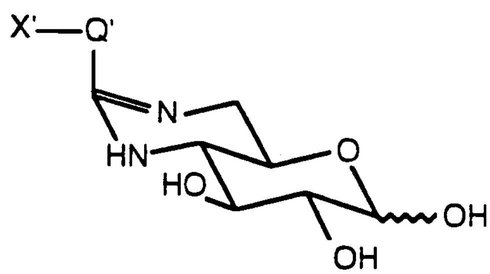


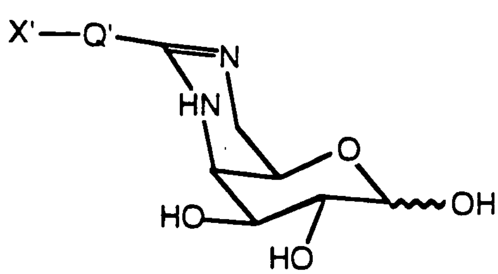
wherein X' is an analog of X of compound C4f, and which is optionally linked to a carrier protein,

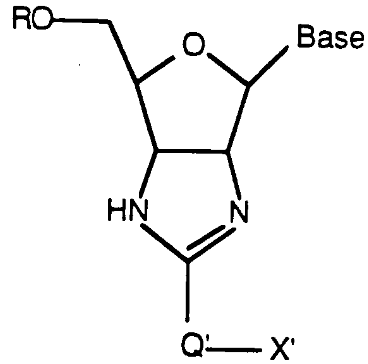
Q' is CH_2 or NH, and

G' is a radical of the diol $G(OH)_2$, $G(OH)_2$ is a sugar, cycloalkyldiol or ortho-phenyldiol, and G' is optionally substituted with halogens, heteroatoms, phosphonate, sulfonate, carboxylate, alkylammonium, alkene, or monocyclic aromatic, and is optionally linked to a carrier protein.

Examples of the above are as follows:





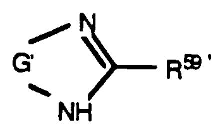


Base = Uracil, 5-Fluorouracil, Cytosine, Adenine, Guanine or analogues thereof

R = H, PO_3H_2

Sugar orthoester hapten 2

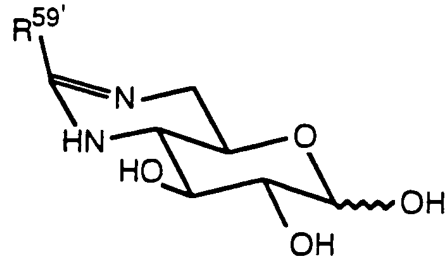
Useful as a hapten as well as a prodrug is a compound C4h having the formula:

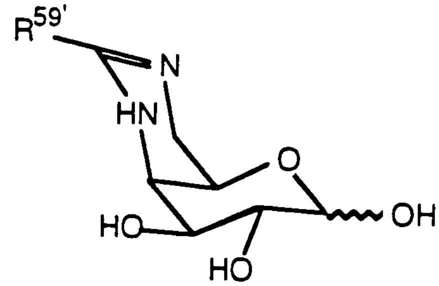


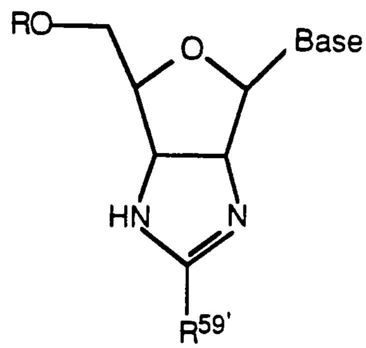
G' is a radical of the diol $G(OH)_2$, $G(OH)_2$ is a sugar, cycloalkyldiol or ortho-phenyldiol, and G' is optionally substituted with halogens, heteroatoms, phosphonate, sulfonate, carboxylate, alkylammonium, alkene, or monocyclic aromatic, and is optionally linked to a carrier protein, and

$R^{59'}$ is H, alkyl unsubstituted, alkyl substituted with halogens, heteroatoms, phosphonate, sulfonate, carboxylate or alkyl ester or alkyl amide, hydroxyl, alkylammonium, amino, alkene, or monocyclic aromatic, and is optionally linked to a carrier protein.

Examples of the above are as follows:





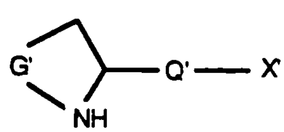


Base = Uracil, 5-Fluorouracil, Cytosine, Adenine, Guanine or analogues thereof

R = H, PO_3H_2

Sugar orthoester hapten 3

Useful as a hapten as well as a prodrug is a compound C4i having the formula:

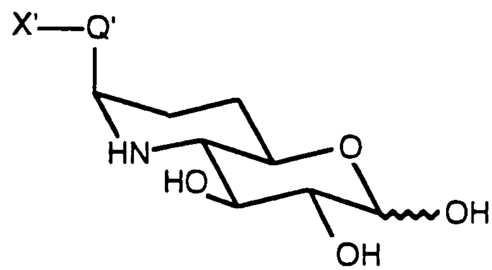


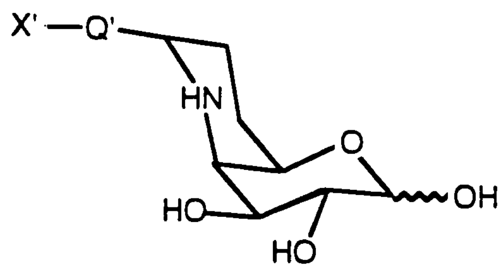
wherein X' is an analog of X of compound C4f, and which is optionally linked to a carrier protein,

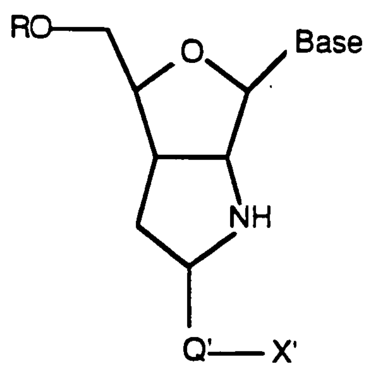
Q' is CH_2, and

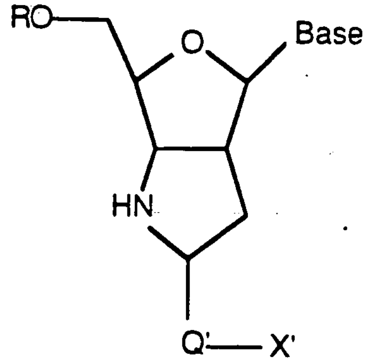
G' is a radical of the diol $G(OH)_2$, $G(OH)_2$ is a sugar, cycloalkyldiol or ortho-phenyldiol, and G' is optionally substituted with halogens, heteroatoms, phosphonate, sulfonate, carboxylate, alkylammonium, alkene, or monocyclic aromatic, and is optionally linked to a carrier protein.

Examples of the above are as follows:







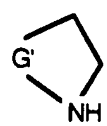


Base = Uracil, 5-Fluorouracil, Cytosine, Adenine, Guanine or analogues thereof

R = H, PO_3H_2

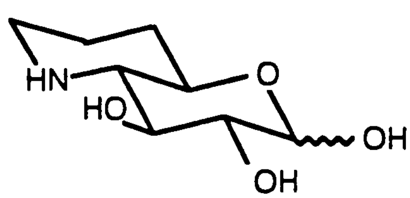
Sugar orthoester hapten 4

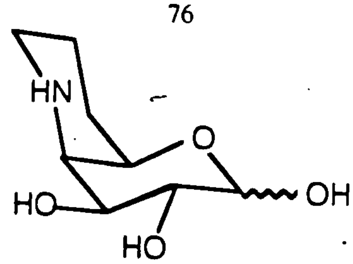
Useful as a hapten as well as a prodrug is a compound C4 having the formula:

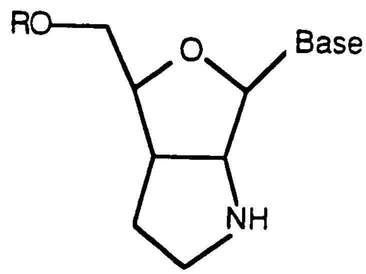


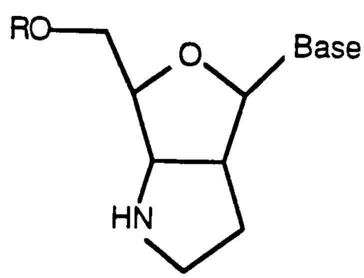
G' is a radical of the diol $G(OH)_2$, G(OH)2 is a sugar, cycloalkyldiol or ortho-phenyldiol, and G' is optionally substituted with halogens, heteroatoms, phosphonate, sulfonate, carboxylate, alkylammonium, alkene, or monocyclic aromatic, and is optionally linked to a carrier protein.

Examples of the above are as follows:









Base = Uracil, 5-Fluorouracil, Cytosine, Adenine, Guanine or analogues thereof

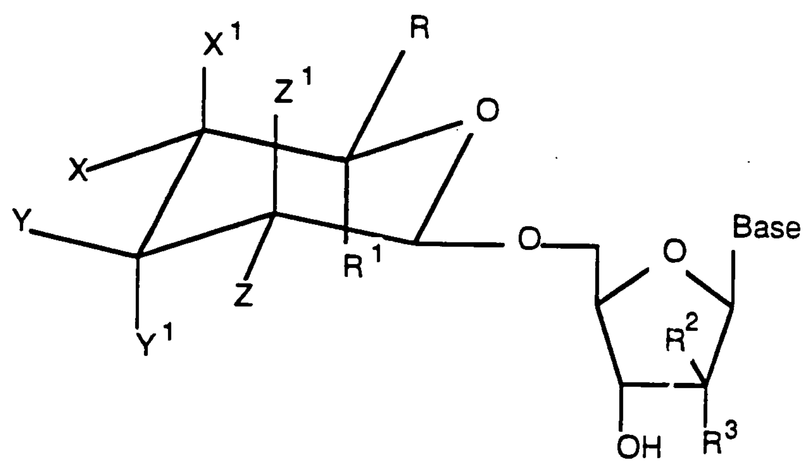
R = H, PO_3H_2

Glycosidase Catalysis

Novel compounds in accordance with the invention are prodrugs of an antineoplastic nucleoside analog (or other antineoplastic agent) comprising a monosaccharide hexopyranose or hexofuranose covalently attached via the anomeric position to the 3' or 5' oxygen of the nucleotide analog, in particular such prodrugs wherein said hexopyranose or hexofuranose is selected from the group consisting of glucose, glucosamine, D-quinovopyranose, galactose, galactosamine, L-fucopyranose, L-rhamnopyranose, D-glucopyranuronic acid, D-galactopyranuronic acid, D-mannopyranuronic acid, or D-iodopyranuronic acid.

The haptens for a glycosyl prodrug of an antineoplastic nucleoside analog comprise an amidine analog of a monosaccharide hexopyranose or hexofuranose in which the nucleoside oxygen of attachment is replaced by NR^1 and the furanose or pyranose ring oxygen is replaced by NR^2. Such haptens include amidine analogs of a monosaccharide hexopyranose or hexofuranose which is a structural analog of a sugar selected from the group consisting of glucose, glucosamine, D-quinovopyranose, galactose, galactosamine, L-galactopyranuronic acid, D-mannopyranuronic acid, or D-iodopyranuronic acid.

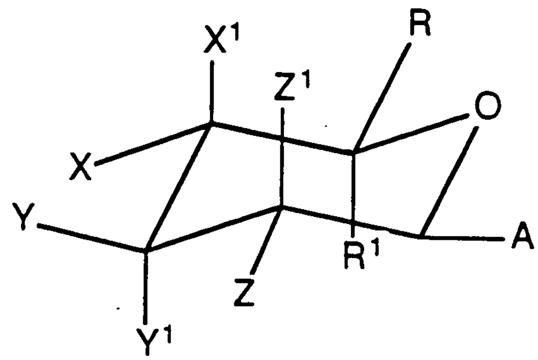
The compounds include compounds of the formula:



R^2 and R^3 are H or OH but only one can be OH; X, X^1, Y, Y^1, Z, Z^1, R and R^1 are as defined in the table below.

A novel coupling reaction to make β-glycosylated nucleosides of the invention from hexopyranoses and nucleosides is the direct reaction of the peracetylated hexoses and the 5' hydroxy nucleosides in the presence of a Lewis acids such as TMS triflate, BF_3Et_2O etc. in the solvent, acetonitrile. This method can be extended to the sugars listed below to make the corresponding β-glycosylated nucleosides.

The coupling reaction can be accomplished also by activation of the anomeric position by conversion to SPh, F or imidate groups and subsequent reaction with the 5' hydroxy nucleosides to make the corresponding glycosylated nucleosides.

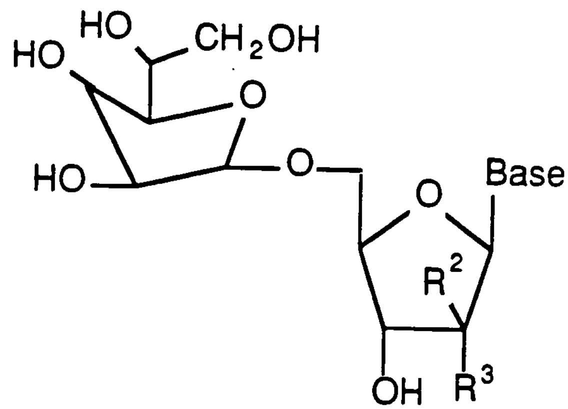


X, X^1, Y, Y^1, Z, Z^1, R, R^1 and A are as defined in the table below.

A = OAc, SPh, F, Imidate

Name of the sugar	X^1	X	Y^1	Y	Z^1	Z	R	
	R^1							
Glucose	H	OH	H	OH	H	OH	CH_2OH	H
Glucosamine	H	OH	H	OH	H	NH_2	CH_2OH	H
D - Quinovopyranose	H	OH	H	OH	H	OH	CH_3	H
Galactose	OH	H	H	OH	H	OH	CH_2OH	H
Galactosamine	OH	H	H	OH	H	NH_2	CH_2OH	H
L-Fucopyranose	OH	H	H	OH	H	OH	CH_3	H
L- Rhamnopyranose	H	OH	H	OH	H	OH	CH_3	H
Hexuronic Acids :								
D Glucopyranuronic acid	H	OH	H	OH	H	OH	COOH	H
D Galactopyranuronic acid	OH	H	H	OH	H	OH	COOH	H
D manopyranuronic acid	H	OH	H	OH	OH	H	COOH	H
D Iodopyranuronic acid	H	OH	H	OH	H	OH	H	COOH

The coupling reaction of hexofuranoses at their anomeric position to the nucleoside 5' position to make furanosylated nucleosides can be accomplished by the method described above.



wherein R^2 = H and R^3 = OH or R^2 = OH and R^3 = H.

Coupling of hexofuranoses to nucleosides will make a mixture of anomers, because of the ring size.

D. Prodrug Activation By Glycosidase Reaction

1. **Hexopyranose conjugated to drug hydroxyl group via the anomeric position of the sugar**
2. **Hexofuranose conjugated to drug hydroxyl group via the anomeric position of the sugar.**

Glycosidase prodrug

Included in the invention is a compound D1a having the formula:

V-Q-X

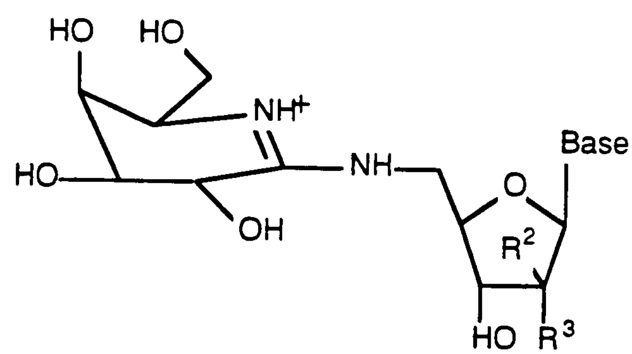
wherein X is a radical of the drug XQH. Advantageously, XQH is a cytotoxic drug such as a nucleoside analog or phosphoramide mustard [$HOP(O)(NH_2)N(CH_2CH_2Cl)_2$], melphalan or doxorubicin.

Q is O or NH, and

V is a hexopyranose or hexofuranose conjugated to QX via the anomeric position of the sugar with optional alpha or beta configuration.

Advantageously V is Glucose, Glucosamine, D-Quinovopyranose, Galactose, Galactosamine, L-Fucopyranose, L- Rhamnopyranose, D-Glucopyranuronic acid, D-Galactopyranuronic acid, D-manopyranuronic acid, or D-Iodopyranuronic acid.

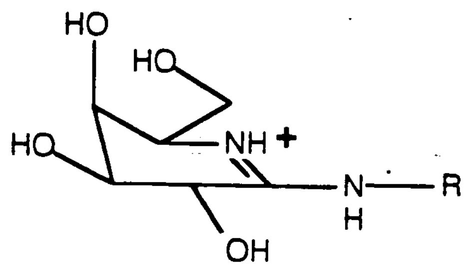
Amidine haptens are prepared as transition-state analogs for eliciting an immune response to make catalytic antibodies. The amidine hapten mimics the transition state for the hydrolysis of the glycosidic bond. Because of the sofa/chair conformation of the hapten, antibodies raised to these haptens may cleave a wide variety of monosaccharide hexopyranoses.



wherein $R^2 = H$ and $R^3 = OH$ or $R^2 = OH$ and $R^3 = H$.

The synthesis of the haptens is accomplished by the coupling reaction of the appropriate lactam and the corresponding 5 amino nucleoside in the presence of triethyloxonium tetrafluoroborate in methylene chloride as the solvent .

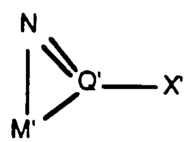
A hapten (amidine TS analog) for galactose or equivalent sugar for the cleavage of the glycosidic bond to liberate drug, said hapten having the formula:



R= Nucleoside, Sugar, or any equivalent drug

Glycosidase hapten 1

Useful as a hapten as well as a prodrug is a compound D1b having the formula:



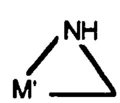
wherein X' is an analog of X of compound D1a, and which is optionally linked to a carrier protein,

Q' is NH, and

M' is a 1,4-diradical of a n-pentane where C1, C2, C3 and C5 are optionally substituted with OH, and M' is optionally linked to a carrier protein.

Glycosidase hapten 2

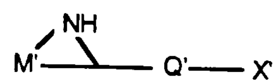
Useful as a hapten as well as a prodrug is a compound D1c having the formula:



M' is a 1,4-diradical of a n-pentane where C1, C2, C3 and C5 are optionally substituted with OH, and M' is optionally linked to a carrier protein.

Glycosidase hapten 3

Useful as a hapten as well as a prodrug is a compound D1d having the formula:



wherein X' is an analog of X of compound D1a, and which is optionally linked to a carrier protein,

Q' is CH_2, and

M' is a 1,4-diradical of a n-pentane where C1, C2, C3 and C5 are optionally substituted with OH, and M' is optionally linked to a carrier protein.

Prodrugs of Nucleoside Analogs

A number of cytotoxic nucleoside analogs have utility as antitumor agents, though there is often a low margin of safety. Effective antineoplastic doses of these drugs can have serious side effects, generally related to their toxicity toward normal tissues such as bone marrow or gastrointestinal mucosa.

5-Fluorouracil (5-FU) is a major antineoplastic drug with clinical activity in a variety of solid tumors, such as cancers of the colon and rectum, head and neck, liver, breast, and pancreas. 5-FU has a low therapeutic index. The size of the dose that is administered is limited by toxicity, reducing the potential efficacy that would be obtained if higher concentrations could be attained near tumor cells.

5-FU must be anabolized to the level of nucleotides (e.g., fluorouridine- or fluorodeoxyuridine- 5'-phosphates in order to exert its potential cytotoxicity. The nucleosides corresponding to these nucleotides (5-fluorouridine and 5-fluoro-2'-deoxyuridine) are also active antineoplastic agents, and in some model systems are

substantially more potent than 5-FU, probably because they are more readily converted to nucleotides than is 5-FU.

The methods for localized delivery of fluorouridine to tumor cells of the subject invention have the advantage of providing high concentrations at the tumor site(s) with minimal systemic exposure. Another degree of tumor selectively is obtained through the rapid catabolism of fluorouridine (to form, initially, the less toxic 5-FU) that is not immediately taken up by tumor cells.

Similarly, arabinosylcytosine (Ara-C) is widely used in treating leukemias and lympohomas. Ara-C is rapidly degraded by cytidine deaminase, producing the inactive metabolite arabinosyluracil. Therapeutic use of Ara-C often results in side effects related to bone marrow suppression or damage to gastrointestinal mucosa. Targeted delivery of Ara-C, e.g., into lymphomas, results in increased therapeutic efficacy with minimized side effects.

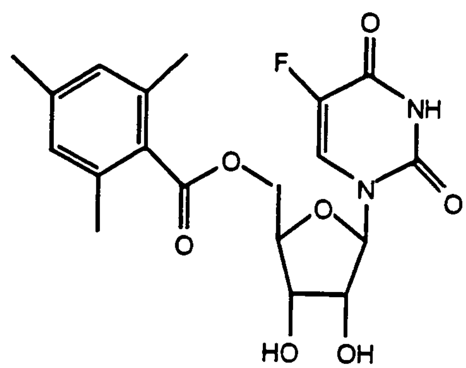
A similar argument and rationale holds true for other antineoplastic nucleoside analogs, including but not limited to: fluorouracil arabinoside, mercaptopurine riboside, 5-aza-2'-deoxycytidine, arabinosyl 5-azacytosine, 6-azauridine, azaribine, 6-azacytidine, trifluoro-methyl-2'-deoxyuridine, thymidine, thioguanosine, 3-deazauridine.

In the present invention, prodrugs of antineoplastic nucleoside analogs are made by attaching an appropriate substituent to the 5' position of the aldose ring. A substituent in this position reduces toxicity of the drug, since cytotoxic nucleoside analogs must typically be phosphorylated (yielding a nucleotide analog) in order to manifest their toxicity. Substituents on the 5' position also render nucleoside analogs stable to the nucleoside-degrading enzymes uridine phosphorylase (which degrades uridine and analogs thereof) and cytidine deaminase (which degrades cytidine and analogs thereof). Prodrugs with substituents on the 3' position

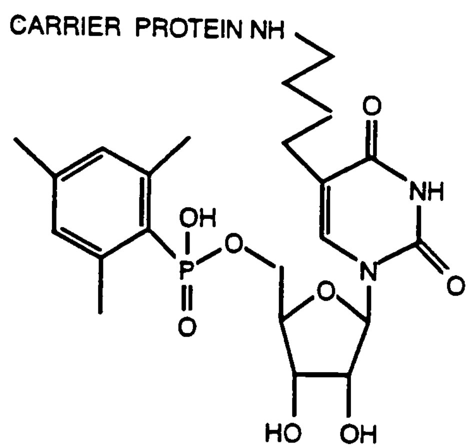
of the aldose ring of antineoplastic nucleoside analogs are also useful for targeted delivery of antineoplastic nucleoside analogs.

Examples of nucleoside analog prodrugs and haptens are as follows:

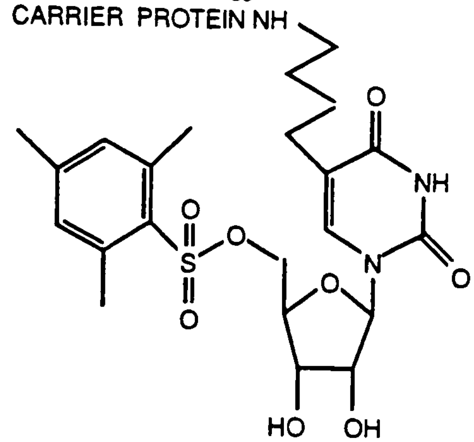
5-Fluorouridine-5'-O-2,4,6 trimethylbenzoate



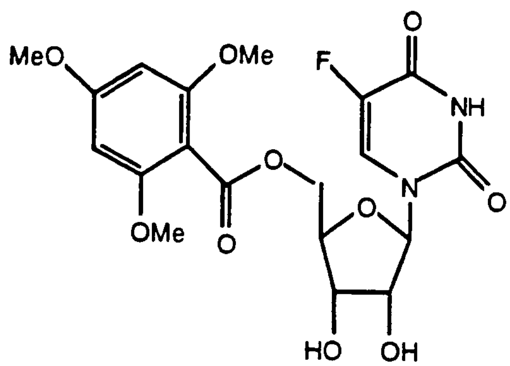
Phosphonate hapten for 5-fluorouridine-5'-O-2,4,6 trimethylbenzoate



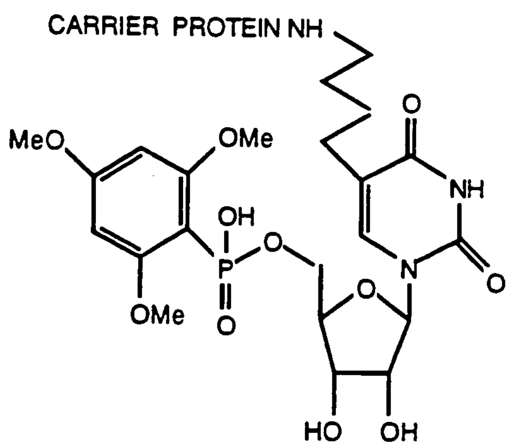
Sulphonate hapten for 5-fluorouridine-5'-O-2,4,6 trimethylbenzoate



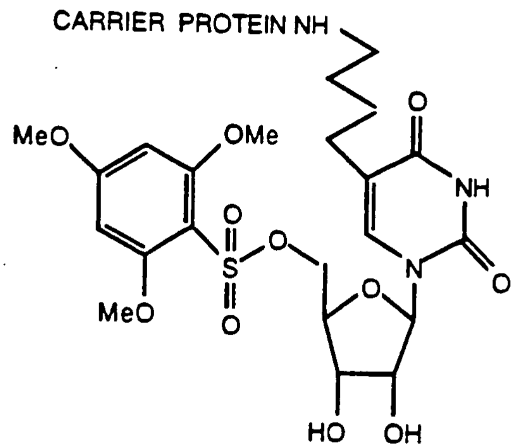
5-Fluorouridine-5'-O-2,4,6 trimethoxybenzoate



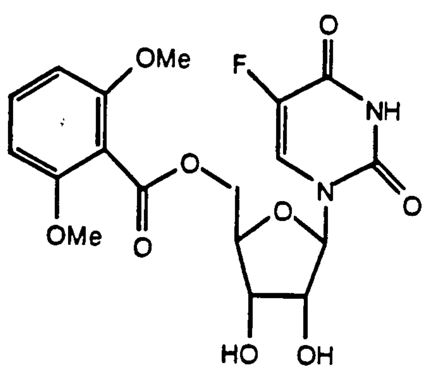
Phosphonate hapten for 5-fluorouridine-5'-O-2,4,6 trimethoxybenzoate



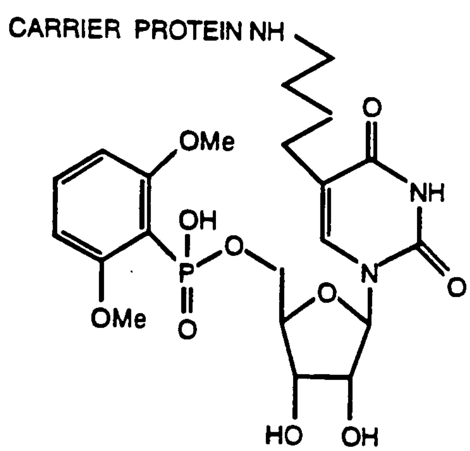
Sulphonate hapten for 5-fluorouridine-5'-O-2,4,6 trimethoxybenzoate



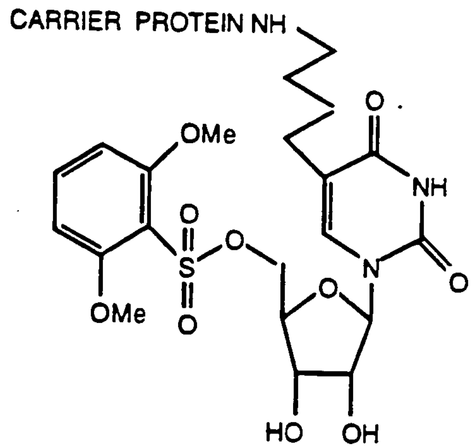
2,6-dimethoxybenzoate



Phosphonate hapten for 5-fluorouridine-5'-O-2,6 dimethoxybenzoate



<u>Sulphonate hapten for 5-fluorouridine-5'-O-2,6 dimethoxybenzoate</u>



<u>Prodrugs Of Alkylating Agents</u>

The present invention also provides novel methods and compounds for achieving localized delivery and formation of active alkylating agents.

Prodrug substituents of the invention, a...ned to certain cyclophosphamide metabolites (e.g., 4-hydroxycyclophosphamide or aldophosphamide) prevents their enzymatic and chemical breakdown to cytotoxic products. An appropriate protein catalyst, conjugated to a tumor-selective reagent, is administered prior to the prodrug; the catalyst thereupon produces active alkylating species in the vicinity of tumor cells after subsequent administration of the prodrug.

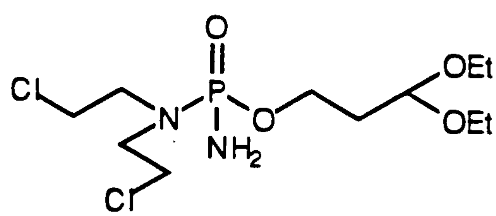
The present invention utilizes prodrugs related to cyclophosphamide, which is the most widely used alkylating agent in clinical practice, with utility in treating cancers of breast, endometrium, and lung, as well as in treating leukemias and lymphomas. Cyclophosphamide, as such, is inactive and is converted primarily in the liver to 4-hydroxycyclophosphamide, which then breaks down further into cytotoxic metabolites. Thus, after cyclophosphamide administration, the active metabolites of cyclophosphamide are spread systemically via the circulation following release from the liver and cannot be concentrated in the area of tumor cells by, for example, localized injection. The active

cytotoxic metabolites of cyclophosphamide are unstable or very toxic and thus, cannot be administered directly. Side effects of cyclophosphamide treatment include leukopenia, bladder damage, and alopecia. The present invention provides methods and compounds for providing suitable prodrugs of cytotoxic cyclophosphamide metabolites that are activated in one embodiment of the invention by catalytic antibodies.

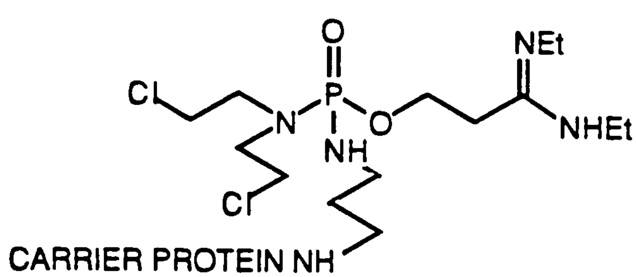
Similar prodrugs and haptens related to other alkylating agents are within the scope of the invention. Other antineoplastic alkylating agents include but are not limited to alkyl sulfates such as busulfan, aziridines such as benzodepa or meturadepa, nitrosoureas such as carmustine, and nitrogen mustards such as chlorambucil, melphalan, ifosfamide or mechlorethamine.

Examples of aldophosphamide prodrugs and haptens are as follows:

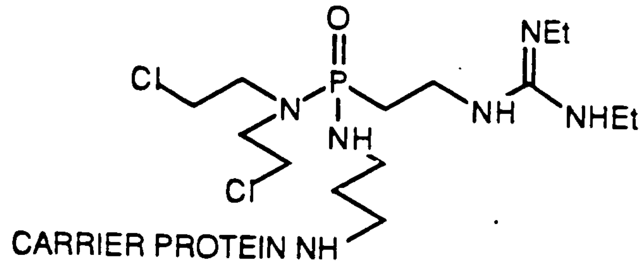
Aldophosphamide-diethylacetal



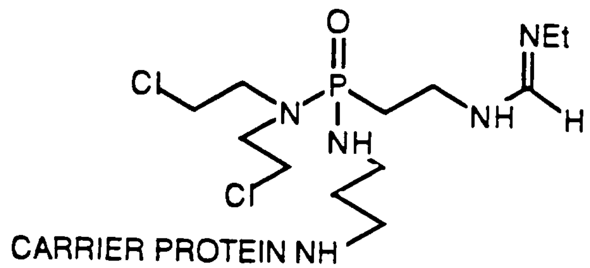
Hapten for aldophosphamide-diethylacetal



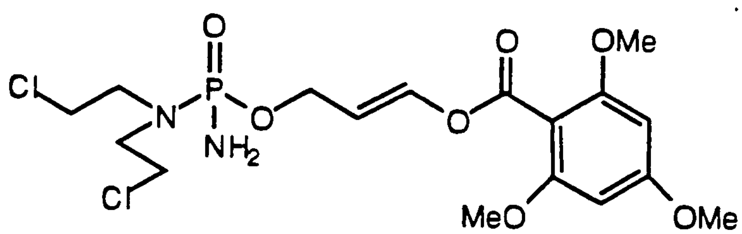
Hapten for aldophosphamide-diethylacetal



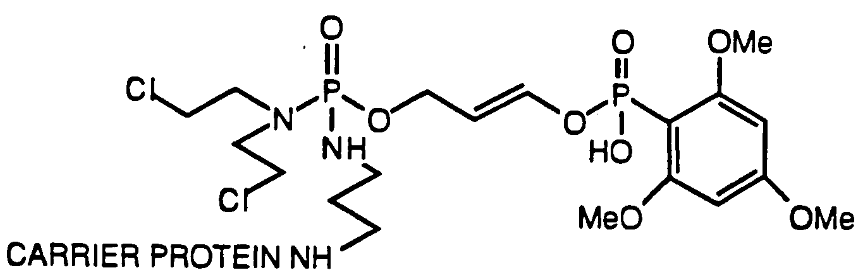
Hapten for aldophosphamide-diethylacetal



2,4,6-Trimethoxybenzoate ester of enol form of aldophosphamide

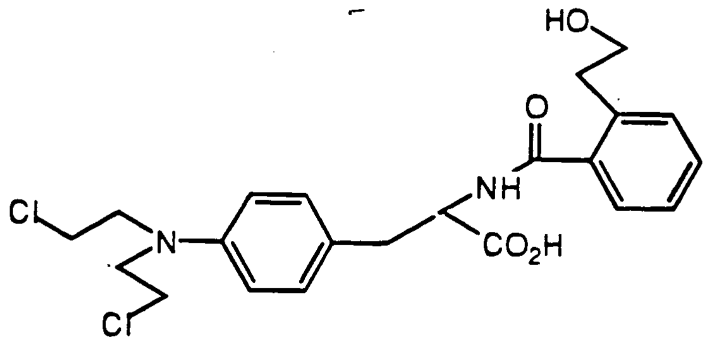


Phosphonate hapten for 2,4,6-Trimethoxybenzoate ester of enol form of aldophosphamide

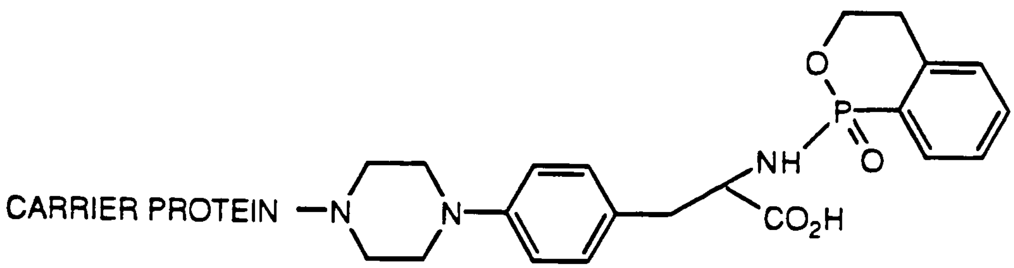


Examples of melphalan prodrugs and haptens are as follows:

Melphalan-2-hydroxyethyl benzoic acid amide



Phosphonate hapten for melphalan-2-hydroxyethyl benzoic acid amide



Prodrugs of Other Antineoplastic Agents

Prodrugs of a wide variety of antineoplastic agents are prepared by their conjugation to prodrug substituents of the invention. Ester or glycosyl substituents of the invention are appropriate for drugs with hydroxyl groups; amide substituents are appropriate for drugs containing amino groups (particularly primary amino groups); acetal substituents are appropriate for drugs containing aldehyde groups.

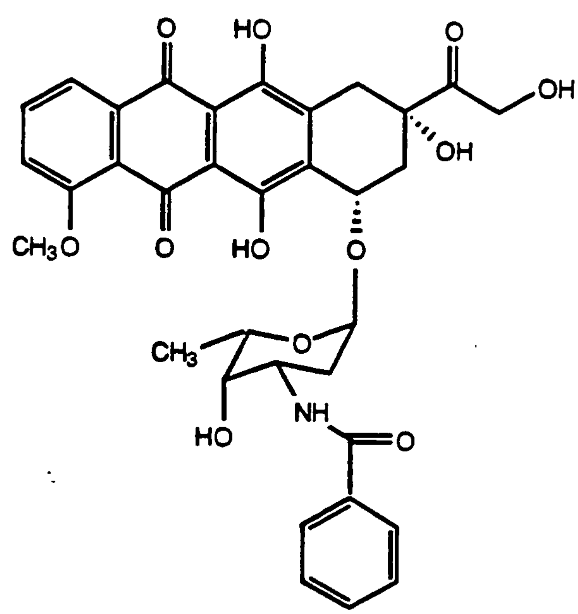
Doxorubicin and related anthracycline antineoplastic agents like daunorubicin and epirubicin are suitable drugs for targeted delivery using the methods of the invention. The primary amino group on the daunosamine ring of this class of drugs is a good site for attachment of one of the amide substituents of the invention, and the hydroxyl groups on either the daunosamine ring or the aglycone moiety are suitable for attachment of an ester substituent of the invention. Such substituents reduce the cytoxicity of the anthracycline drugs; cytotoxicity is restored at the tumor site by an appropriate targeted catalytic protein.

Similarly, other antineoplastic drugs that are suitable for targeted delivery using the methods of the invention, include but are not limited to: folate antagonists like methotrexate or trimetrexate; podophyllin compounds like etoposide or teniposide, Vinca alkaloids like vincristine, vinblastine or vindesine; tubulin modifiers like taxol, antibiotics like dactinomycin, and bleomycins.

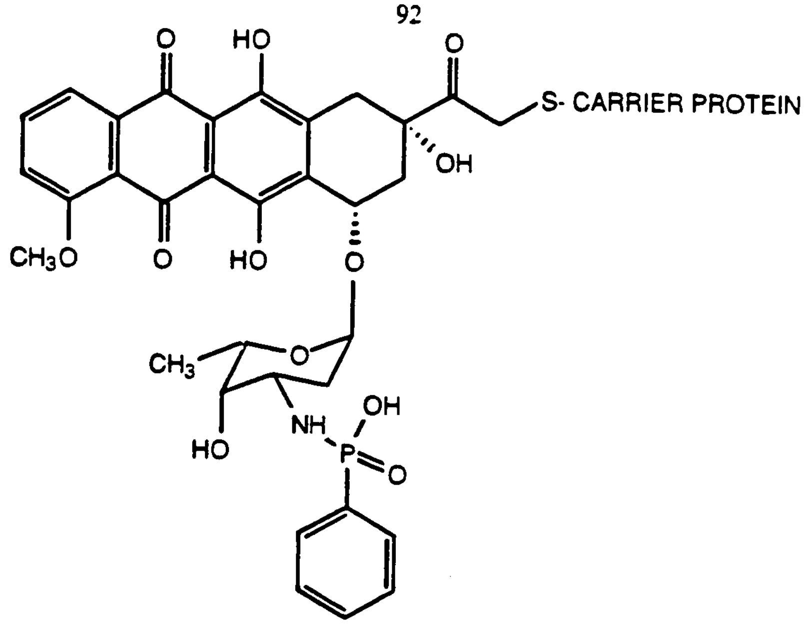
In addition, cytotoxic drugs which are not in themselves useful as antineoplastic agents *in vivo*, due to excessive toxicity to normal tissues, can be used as targeted antitumor agents using the methods and prodrug substituents of the invention. Such cytotoxic substances include the trichothecene toxins.

Examples of doxorubicin prodrugs and haptens are as follows:

Doxorubicin-benzoic acid amide



Phosphonate hapten for doxorubicin-benzoic acid amide



Catalytic Proteins for Activating Prodrugs and Targeting the Prodrugs

Catalytic Proteins for Activating Prodrugs

In addition to the development of suitable prodrugs, an appropriate catalytic protein for activation of these prodrugs (i.e. enhancing the rate of cleavage of the drug from the residue of the prodrug) must be selected in this therapeutic strategy .

a. Enzymes for Activating Prodrugs

Enzymes, or active fragments thereof can be used with the novel prodrugs of the subject invention in cases where enzymes with appropriate catalytic activity exist. The enzyme and catalytic activities used in the constructs of the subject invention are selected from: glycosidase, peptidase, lipase (or other hydrolases) oxido-reductase, transferase, isomerase, lyase or ligase.

Examples of enzymes for use with the novel prodrugs of the invention are described below:

A. Esterase - cleaves acyl substituents esterified to drugs

Carboxylesterase (E.C. 3.1.1.1)

Arylesterase (E.C. 3.1.1.2)

Triacylglycerol lipase (E.C. 3.1.1.3)

Acetylesterase (E.C. 3.1.1.6)

Galactolipase (E.C. 3.1.1.26)

Cephalosporin-C deacetylase (E.C. 3.1.1.41)

6-0-Acetylgucose deacetylase (E.C. 3.1.1.33)

lipase

B. Amidase - cleaves acyl substituents attached to amino groups

Peptidases (endo- and exopeptiases)

β-Lactamases (Classes A, B, and C) and Penicillin amidase

Acetylornithine deacetylase (E.C. 3.5.1.16)

Acyl-lysine deacylase (E.C. 3.5.1.17)

C. Acetal hydrolase - hydrolyzes acetals (or ortho esters) to aldehydes

Alkenyl-glycerophosphocholine hydrolase (E.C. 3.3.2.2)

Cellulase (E.C. 3.2.1.4)

Oligo-1, 6-glucosidase (E.C. 3.2.1.10)

Lysozyme (E.C. 3.2.1.17)

β-D-Glucuronidase (E.C. 3.2.1.31)

D. Glycosidase - cleaves sugar substituents attached to drugs via an ether linkage

Examples include beta-galactosidases, beta-glucosidases, inulases, alpha-L-arabinofuranosidases, agarases, and isomerases. Specific examples include:

β-D-Glucosidase (E.C. 3.2.1.21)

α-D-Glucosidase (E.C. 3.2.1.20)

β-D-Galactosidase (E.C. 3.2.1.22)

α-D-Galactosidase (E.C. 3.2.1.23)

β-D-Fructofuranosidase (E.C. 3.2.1.26)

α,α-Trehalase (E.C. 3.2.1.28)

α-L-Fucosidase (E.C. 3.2.1.51)

Glycosylceramidase (E.C. 3.2.1.62)

Lyases can be used with prodrugs which also serve as haptens.

The primary aim is to select an enzyme activity not normally present in the serum or other body compartments to which the drug is exposed, which is capable of activating the prodrug and does not cause significant damage to normal physiological compounds or macromolecules. Enzymes for use with the prodrugs can be selected using screening techniques such as those described below for catalytic antibodies

b. Antibodies for Activating Prodrugs

The catalytic antibodies or active fragments thereof used in the subject invention are those of the prior art (see the section above on catalytic antibodies in Background of the Invention) and those made using the novel haptens described herein (see the section above entitled Novel Prodrugs and Haptens of the Invention) with the techniques known to those skilled in the art for making catalytic antibodies. See U.S. Patents 4,963,355, 4,888,281 and 4,792,446 hereby incorporated herein by reference.

Target Reagents

The targeting component of the targeting and activating compounds of the invention includes any agent which selectively binds or concentrates on or in the vicinity of a specific cell population for example, any antibody or other compound which binds specifically to a tumor-associated antigen (other examples include hormones, growth factors, substrates, or analogs of enzymes, etc.). Examples of such antibodies include, but are not limited to, those which bind specifically to antigens found on carcinomas, melanomas, lymphomas and bone and soft tissue sarcomas as well as other tumors. Antibodies that remain bound to the cell surface for

extended periods or that are internalized very slowly are particularly advantageous. These antibodies are polyclonal or advantageously, monoclonal, and are intact antibody molecules or fragments containing the active binding region of the antibody.

The system, according to the invention, is used for delivering a drug at any host target site where treatment is required, providing the target site has one or more targetable components, for example, epitopes that are substantially unique to that site and which are recognized and bound by the immunoconjugate. Particular target sites include those regions in a host arising from a pathogenic state induced by, for example, a tumor, a bacterium, a fungus or a virus; or as a result of a malfunction of a normal host system, for example, in cardiovascular diseases, such as the formation of the thrombus, in inflammatory diseases, and in diseases of the central nervous system.

The use of genetic cloning and engineering methods have revolutionized the potential to generate reagents able to target an enzyme or catalytic antibody. This has been exemplified by the progress which has occurred in the area of immunology.

a. Antibodies Which Bind Tumor Cells

Advantageously, antibodies which bind antigens that are expressed in high density on tumor cells and that do not shed from the tumor are used in the subject invention. These prerequisites are identical to those used in the related field of tumor imaging and treatment using radiolabelled monoclonal antibodies.

A large number of monoclonal antibodies labelled with a variety of radionuclides, including ^{125}I, ^{131}I, ^{111}In, ^{99}mTc, ^{186}Re, ^{90}Y have been used to visualize tumors. This work has shown that a variety of tumors can be successfully visualized by radio-immunoscintigraphic techniques. The tumor types that have been successfully targeted are listed in the table below. Antibodies to the listed antigens for example, are useful to target the prodrug activation.

Tumor Type	Tissue	Mab	Antigen	Reference
Carcinoma	G.I.tract with hepatic metastasis	NR-LU-10	40kD glycoprotein	Goldrosen, M., et al., Cancer Research 50 (1990):7973-7978
Adenocarcinoma	G.I. tract and other tissues	FO23C5	Carcino-embryonic antigen (CEA)	Siccardi, A., Cancer Research 50 (1990):899s-903s
Carcinoma	Head/Neck and Vulva	E48	Peptide epitope within 22kD surface antigen	Gerretsen, M., et al., British Journal of Cancer 63 (1991):37-44
Carcinoma	Larynx, pharynx and parotid gland		CEA	Kairemo, K., et a., Acta Oncoloica 29 (1990):539-543
Carcinoma	Liver	NP-4	CEA	Wang, Z., et al., Cancer Research 50 (1990):869s-872s
Carcinoma	Breast		CEA	Kairemo, K., et al., Acta Oncologica 29 (1990):533-538
Carcinoma	Bladder	BW 431/26	CEA	Boekmann, W., et al., British Journal of Cancer 62 (1990):81-84
Carcinoma	Ovary	HMFG1	Milk fat blobule glyco-protein (>200kD)	Hird, V., et al., British Journal of Cancer 50 (1990):48-51
Carcinoma	Pancreas	DU-PAN1	Glycoprotein expressed in >50% of pancreatic tumors	Worlock, A., et al., Cancer Research 50 (1990):7246-7251
Melanoma	Xenograft in nude mouse	G7A5	High molecular weight-melanoma associated antigen (HMW-MAA). Gp 220 core protein of chondroitin sulfate proteoglycan (250-280kD).	Le Doussal, J. M., et al., Cancer Research 50 (1990):3445-3452

Tumor Type	Tissue	Mab	Antigen	Reference
Melanoma	Lymph node	225.28S and 763.24T	HMW-MAA (different epitopes)	Wahl, R., et al., Cancer Research 50 (1990):941s-948s
Glioma	Brain			Williams, J., et al., Cancer Research 50 (1990):974s-979s
Glioma	Brain	EGFR1	External domain of human and rat epidermal growth factor receptor	Kalofanos, H., et al., J. Nuc Med 30 (1989):1636-1645
		H17E2	Placental alkaline phosphatase (67kD)	
Germ-cell (seminoma and non-seminoma)	Testis	H17E2	Placental alkaline phosphatase (67kD)	Pectasides, D., et al., British Journal of Cancer 62 (1990):74-77

In some cases the use of subfragments of antibodies e.g., F(ab')2 has yielded enhanced specificity of tumor imaging when there has been shown to be a lower actual antigen concentration at the tumor site (Worlock, A., et al., Cancer Research 50 (1990):7246-7251; Gerretsen, M., et al., British Journal of Cancer 63 (1991):37-44). Successful imaging has been possible even in patients with significant serum concentration of antigens shed from the tumor (CEA, Boeckmann, W., et al., British Journal of Cancer 62 (1990):81-84).

b. Other Targeting Proteins

In addition to the use of antibodies, any binding species is useful for binding a catalytic protein (be it enzyme or catalytic antibody) to the site of action. Growth factors have been used to deliver toxin molecules (Siegall, et al., Proc. Natl. Acad. Sci. USA 85 (1985):9738-9742; Chaudhary, et al., Proc. Natl. Acad. Sci. USA 84 (1987):4538-4542; Kondo J., et al., Biol. Chem. 263 (1988):9470-9475). Generation of analogous fusions using the growth factors interleukin 6, interleukin 2, transforming growth factor alpha, and others are made by linking enzymes or abzymes using the methods described in the above references. The

incorporation of catalytic antibodies into these is done via the fusion of these growth factors to the end of antibody single chain gene constructs (Patent Application WO 88/01649) or alternatively the growth factors are fused to the front end of such gene constructs (at the 5' end of the gene or amino terminus of the protein). The use of constructs as described in Patent Application EP A 0,194,276 (Neuberger) are also useful to combine catalytic antibody activity and the binding properties of growth factors.

The use of human CD4-Pseudomonas exotoxin fusion has proved effective in the killing of HIV infected cells. The use of such a binding activity from CD4 linked to an enzyme or catalytic antibody allows the use of prodrug therapy directed at treatment of AIDS. The CD4 binds to the gp120 expressed on HIV1 infected cells. The converse of such a construct makes use of gp120-enzyme (or catalytic antibody) fusion to develop an immunosuppression reagent system (Moore et al., Science, 250, (1990):1139). Other binding species which are useable in the subject invention are the integrin family e.g., LAF-1, which can be used to modulate the immune system (Inghirami et al., Science, 250, (1990):682) and the selection family e.g., ELAM, which can be used to target tumors and immune cells (Walz, et al., Science 250 (1990):1132).

Antibodies can also be used to target prodrugs of the invention to certain blood cell types to treat autoimmune disease. Further, cells overproducing hormones can be targeted.

Production of Bispecific Proteins

a. Production of Bispecific Proteins by Chemical Linkage of Enzymes or Catalytic Antibodies to Targeting Proteins

The enzymes of this invention can be covalently bound to the targeting proteins of this invention by techniques well known in the art such as the use of the heterobifunctional cross-linking reagents SPDP (N-succinimidyl-3(2-pyridyldithio)proprionate) or SMCC (succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate [see, e.g., Thorpe, P. E.,

et al., "The Preparation and Cytotoxic Properties of Antibody-Toxin Conjugates." Immunological Rev., 62 (1982):119-58; Lambert, J. M., et al., supra at p. 12038; Rowland,G. F., et al., supra, at pp. 183-84 and Gallego, J., et al., supra, at pp. 737-38].

b. Production of Bispecific Proteins by Recombinant DNA

Fusion proteins comprising at least the antigen binding region of the targeting protein of the invention linked to at least a functionally active portion of an enzyme or catalytic antibody of the invention can be constructed using recombinant DNA techniques well known in the art [see, e.g., Neuberger, M. S., et al., Nature 312, (1984):604-608]. These fusion proteins act in essentially the same manner as the antibody-enzyme conjugates described herein.

Recombinant DNA methods have been used to express antibody genes in mammalian systems (Oi, V. T., et al., Proc. Natl. Acad. Sci. USA 80 (1983):825-829; Neuberger, M. S., EMBO 2 (1983):1373-1378). Further expression and recovery of biologically active immunoglobulin proteins (human IgE Fc fragment) from E. *coli* has been demonstrated (Kenten, J. H., et al., Proc. Natl. Acad. Sci. USA 81 (1984):2955-2960) and expression and recovery of whole active antibody has been demonstrated (Boss, M.A., et al., Nucleic Acids Res. 12 (1984):3791-3799). This was followed by other groups demonstrating the generality of the potential to generate both immunoglobulin binding and effector function activities in E. *coli* (Cabilly, S., et al., Proc Natl. Acad Sci USA 81 (1984):3273-3277; Skerra, A., et al., Science 240 (1988):1038-1040; Better, M., et al., Science 240 (1988):1041-1043). These skills and abilities have also been applied to manipulation of many other genes.

The following are examples of the prodrug targeting reagents. Most of these depend on the ability to clone, manipulate and express genes as described above.

The use of genetically engineered antibodies as outlined above provide a route to a well-defined and reproducible reagent which allow the rapid analysis of the effectiveness of the

various prodrugs. These methods of antibody engineering are exemplified in European Patent Application EP A 0,194,276 (Neuberger) in which the heavy chain gene is truncated by removal of the CH_2 and CH_3 domains, followed by the addition of various genes. Introduction of the required enzymatic activity follows these basic procedures. To achieve the optimized level of enzyme activity, manipulation of the sequences between the antibody and enzyme may be needed. Addition of linker sequences and/or alteration of the fusion site may be needed for this optimization. In addition, to the advantage of a defined antibody-enzyme reagent, the reduced size possible by the removal of the CH_2 CH_3, and the CH_4 in the case if IgE and IgM heavy chains, is valuable.

Generation of antibodies which are bispecific is well-known to the art (Shawler, et al., Immunol. 135 (1985):1530-1535; Kurokawa, T., et al., Bio/Technology 7 (1989):1163-1167). Examples of the functionality of such bispecific antibodies are the tumor specific antibodies which also bind to metal chelates for use in tumor therapy, and also the bispecific antibodies which bind to tumor cells and T cells (Johnson, M. J., et al., Patent Application EP 369566A, 1990; and Gilliland L.K., et al., Patent Application GB 2197323, 1986). Methods for generation of bispecific antibodies consist of chemical methods of separation and recombination of the antibody chains or by the fusion of the two hybridomas to generate so called quadromas. These methods are effective but are prone to generate mixed species and require purification to isolate the desired products.

Generation of smaller binding species has been the goal of much research in antibody engineering. This has led to the development of single chain antibodies, in which the variable (V) region of the two antibody chains are combined into a single molecule using a linker sequence (Patent Application WO 88/01649, Ladner and Bird). This combination of V regions results in expression of a protein which has one of the V regions at the amino terminus and the other V region attached at its COOH terminus via the linker to its amino terminus. This head to tail, head to tail linkage of V regions has been described with both V

light chain - V heavy chain and V heavy chain - V light chain orientations. The utility of these systems has been developed with the addition to single chain antibodies of other proteins (Vijay, et al., Nature 339 (1989):394-397). This format, for the addition of other proteins to the end of single chain antibodies, is used for the production of similar molecules making use of desired enzyme genes to affect these constructions. This results in the production of molecules having the desired properties of antibody binding and enzymatic activity in a small molecule ideally suited for therapy.

To engineer the production of two antibody activities into a bispecific molecule in a single chain or equivalent small molecule follows the outline below using methods well known to those skilled in the art.

The construct would consist of: the V Heavy chain region (VH) linked to the V Light chain region (VL); specific for the tumor cell or antigen via the linkers described for single chain antibodies (Vijay, et al., Nature 339 (1989):394-397; Patent Application WO 88/01649, Ladner and Bird); these sequences are linked directly to the catalytic antibody VL which would, in turn, be linked to its VH partner via a linker sequence. The V region combinations can also follow VL-VH-VH-VL or VL-VH-VL-VH or VH-VL-VH-VL sequences. The linker sequences used in these constructions are those described above for single chain antibody construction. This combination allows the expression of a single chain bispecific antibody previously unknown. Such a molecule allows the production of large amounts of such a bispecific activity without the purification and characterization problems encountered with other methods. This molecule also has the low molecular weight desirable for such a reagent. Another species based on similar construction describes a previously unknown molecule as follows. The VH region specific for the tumor or antigen linked directly to the VL region of the catalytic antibody; this molecule is advantageously expressed separately or together with the other construct of VL specific for the tumor or antigen linked directly to the VH of the catalytic antibody. The expression products of these two molecules together, or by

post expression mixing, associate to form a bispecific antibody. Other combinations of V regions lead to similar molecules. This molecular species is favored over the molecule above as it has a lower molecular weight. The use of single domain binding proteins is also valuable to explore in the form of direct fusions to enzymes or catalytic antibodies (Patent Application WO 90/05144, Winter).

Humanization of Antibodies

Humanization of antibodies and other reagents reduces the immune response. Reagent antigenicity has been a problem in early mouse antibody treatments which have been made ineffective by patients mounting a significant antibody response to the mouse antibodies (Patent Application EP A 0 194 276, Neuberger; Patent Application EP A 0 239 400; LoBuglio, A.F., et al., Proc Natl Acad Sci USA 86 (1989):4220-4224). The immune response is not only associated with the constant regions of the antibody but is also with the variable region domains giving rise to a strong anti-idiotypic response (Bruggemann, M., et al., J. Exp. Med. 170 (1989):2153-2157; Shawler, D. L., et al., Immunol. 135 (1985):1530-1535).

The value of humanization methods for the generation of therapeutically valuable proteins has been demonstrated by humanization of mouse antibodies by replacement of the V-region framework. This humanization method makes use of the basic structure of the binding site with its antigen-binding loops which are fairly well determined (Kabat, E.A., et al., U. S. Dept. of Health and Human Services, U. S. Government Printing Office, 1987). The replacement of the framework with human sequences while retaining the loops from the original antibody effectively transfers the antigen binding from a mouse to a human structural context (Riechmann, L., et al., Nature 332 (1988):323-327; Jones, P. T., et al., Nature 321 (1986):522-524,; Verhoeyen, M., et al., Science 239 (1988):1534-1536; Queen, C., et al., Proc Natl Acad Sci USA 86 (1989):10029-10033). With this humanization technology certain assumptions have been made; A) the contribution of the hypervariable loops to

binding; B) the conservation of framework structure, and that C) the loops all interact with the framework in similar ways. With the use of basic molecular modelling, the humanization can be optimized improving the degree of success (Riechmann, L., et al., Nature 332 (1988):323-327).

These methods aimed at humanization are applicable to enzyme activities. The use of structural analysis allows grafting of homologous outer loop regions in order to camouflage the antigenicity, if enzymes with similar structures to a human protein can be found. Problems of antigenicity can also be obviated by the use of covalent modification i.e., polyethylene glycol modification of the surface of the protein.

Antibody Expression Vectors

Recent advances in the application of PCR cloning of immunoglobulin genes has led to the ability to produce antibody expression libraries in *E. coli* using phage lambda. (Huse, W. D., et al., Science 246 (1989):1275-1281) and the filamentous phage *fd* (Clarkson, T., et al., Nature 352, (1991):624-628). The phage lambda based system generates a library of phage plaques that secrete Fab which can then be screened by a filter binding assay using radiolabeled hapten (Caton, A. J., et al., PNAS, USA 87 (1990):6450-6454). Although potentially valuable for isolating plaques with a desired binding activity, each clone must be individually screened if one is attempting to isolate an antibody mediated catalytic activity. The phage *fd* system for expressing single chain FV antibodies as described in Patent Application WO 92/01047, and incorporated herein by reference describes the production of phage particles that carry antibody FV's fused to the phage gene III protein. This system allows for the direct selection of phage and the genes coding for the specific antibodies expressed on the phage particle by using antibody binding to antigens or haptens. Rare antibodies have been isolated from combinatorial libraries using this method (Marks, J. D., et al., J. Mol. Biol. 222 (1991):581-597).

An alternative approach, not previously described, is to use a plasmid based rather than phage lambda based system for production of the antibody expression library. In this system, rather than having the VH and VL genes as separate transcription units, they are covalently linked by a short peptide to produce a single chain antibody as defined by Bird, E., et al., Science 242 (1988):423-426). Using appropriate PCR primers, a combinatorial single chain antibody library consisiting of essentially random associations of VH and VL is generated by a single step PCR methodology previously described (Davis, G. T., et al., Bio/Technology (1991) in press.). The single-chain PCR product is cloned into a suitable *E. coli* expression vector containing an inducible promotor such as Ptac. A signal sequence, such as pelB, is added 5' of the cloned single-chain to allow secretion of the expressed antibody protein (Better, M., et al., Science 240 (1988):1041-1043). Unlike the phage lambda expression system in which the *E. coli* are lysed, the plasmid based expression system described allows the possibility of directly screening an *E. coli* library for catalytic antibodies using direct selection. One possible selection method, inactivation of a beta-lactam or beta-lactam derivative, is described in the section "Screening for Catalytic Antibodies". Other possible selection methods include antibody catalyzed release of a nutrient, vitamin or cofactor essential for the growth of the *E. coli*. One such selection procedure utilizing thymidine requiring auxotrophs is described in section Screening for Catalytic Activation of Nucleoside Analogue Prodrugs, herein.

VH and VL domains from *E. coli* clones that express antibody with a desired binding or catalytic activity can be mutagenized to alter or enhance antibody function. The specific CDR amino acid residue(s) to be targeted for mutagenesis can be identified by molecular modelling of the antibody active site. Mutagenesis is accomplished by one of a variety of previously described site-directed mutagenesis procedures using mutagenic oligonucleotides (Maniatis, T., et al., Molecular Cloning: A Laboratory Manual, (1989):15.51-15.65, New York: Cold Spring Harbor Laboratory).

If selective mutagenesis is not able to produce the desired result, more extensive alterations of the active site are made. One useful methodology is replacement of one, few or several CDRs with sets or partial sets of random amino acids. This random mutagenesis procedure was successfully used to alter the activity of a beta-lactamase enzyme (Dube, D. K., et al., Biochemistry 28 (1989):5703-5707; Oliphant, A. R., et al., PNAS, USA 86 (1989):9094-9098). The method described involved introduction of random amino acids into the enzyme active site by replacement of the DNA sequence encoding that portion of the active site with a random oligonucleotide.

Random mutagenesis of an antibody CDR region is accomplished by any of a number of different methods. One example of a protocol that is used to randomly mutagenize CDR1 VH of an anti-fluorescein monoclonal antibody (Mab 4-4-20, Bedzyk, W. D., et al., JBC 264 (1989):1565-1569) is presented in detail below.

1. An oligonucleotide of the following sequence shown below is synthesized on an automated DNA synthesizer. The number above certain nucleotide triplets corresponds to the amino acid position within 4-4-20 VH as designated by Bedzyk, et al., (1989).

```
  21              25           30              (CDR1)          35
5'TCC TGT GTT GCC TCT GGA TTC ACT TTT AGT (NNKNNKNNKNNK) AAC TGG

                               40
          GCT CGC CAG TCT CCA GAG AAA GGA-3'
```

In the sequence above (SEQ ID NO:1), VH CDR1 (amino acids 31-34) is replaced with a random nucleotide sequence where N is A, C, G, or T (equimolar) and K is G or T (equimolar). Excluding A or C at the third position in each triplet will reduce the number of potential termination codons by two thirds as reported by Cwirla, S. E., et al., PNAS, USA 87 (1990):6378-6382.

2. A second oligonucleotide is synthesized which is complimentary to the last 20 base pairs at the 3' end of the oligonucleotide from Step 1. Following phosporylation with T4 kinase, oligonucleotides are annealed and then added to a primer extension reaction containing deoxynucleotides and Klenow fragment. The resulting full length double stranded random oligonucleotide is purified by polyacrylamide gel electrophoresis or reverse phase HPLC.

3. Double stranded random oligonucleotide from Step 2 can serve as a "sticky foot" primer in the "sticky foot" mutagenesis procedure described by Clackson, T., et al., NAR 17 (1989):10163-10170. This procedure will result in replacement of the wild type VH CDR1 present in the template strand with a random CDR1 sequence specified by the random oligonucleotide described in Step 1.

4. Following sticky foot mutagenesis the DNA from Step 3 is used to transform *E. coli* resulting in an antibody library in which VH CDR1 is replaced with a random sequence.

5. The resulting library can be screened by binding assays with appropriate hapten or selection assays as described in preceding sections of the patent.

Additional CDR regions of either VH or VL can be randomly mutagenized in a similar fashion. In addition, one, two, or all three CDR regions within a VH or VL chain can be mutagenized simultaneously. Due to limitations on the length of an oligonucleotide that can be synthesized on an automated machine, 3 separate random oligonucleotides corresponding to each of the 3 CDR regions can be made as described in Step 1 above. During oligonucleotide synthesis, restriction sites are incorporated at appropriate positions within framework regions that flank each of the CDRs. Following conversion into double stranded DNA as in Step 2 above, each oligonucleotide is digested with the appropriate restriction

enzyme and the oligonucleotides are ligated together to produce a complete VH or VL. The final ligated product is then used as a "sticky foot" primer as in Step 3 above.

An alternative approach to the method described above is to engineer restriction enzyme sites into the framework regions on each side of the CDR VH or VL to be mutagenized. During synthesis of the random oligonucleotide as in Step 1 above, compatible restriction sites are then added to the framework flanking regions. Restriction sites are chosen so as to best preserve the wild type coding sequence within the framework region. The wild type CDR region is then removed by digesting with the appropriate restriction enzyme and replaced with the double stranded random oligonucleotide digested with compatible restriction enzymes.

Selection of New Binding Activities Using Mutagenesis and Selection in Filamentous Phage

Selection of mutant antibodies by selection for growth under selective conditions has been illustrated (see the section entitled, "Screening of Mutant Catalytic Antibodies in *E. coli*). In concert with these methods for selection and mutagenesis, the use of methods described by Cwirla S., et al., PNAS, 87 (1990):6378-6382; and McCafferty J., et al., Nature, 348 (1991):552-554 help generate/improve catalytic antibodies for prodrug activation.

These methods have allowed for the generation of vast libraries of peptides and the screening via the binding of the resultant mutants by taking the mutant single chain antibodies generated in the protocol as outlined above and inserting these into the adsorption protein (gene III) of the filamentous bacteriophage, fd. The site for the introduction of the PCR cloned and mutagenized single chain antibody is 5-6 amino acids from the N terminus of the adsorption protein (gene III). This allows for the presentation of the antibody for binding to antigen. The vector (fd-CAT1) after insertion of the single chain antibody gene is then used to electrotransform *E. coli* TG1 (K12, (lac-pro), supE, thi, hsdD5/F traD36, proA+B+ lacIq, lacZM15) or similar host.

The transformed *E.coli* are then subjected to selection using the tetracycline resistance of the vector. This phage library is cultured on plates allowing its amplification and the estimation of the library size (library sizes in the range of 10^{12} allow the screening of random mutants at 9 sites in the antibody).

This library is then subjected to amplification in liquid culture the resultant phage in the supernatant are concentrated using polyethylene glycol precipitation and dissolved in PBS with 2% skimmed milk powder. These phage are then mixed with, for example 100 μl of solid phase-antigen, such as epoxy activated Sepharose CL-6B (Sigma Ltd) reacted with a suitable antigen, for the selection of the desired binding activity. The candidate compounds for use in this selection would include those haptens described herein. These antigens used for the raising of antibodies can also be coupled indirectly to a solid phase, such as epoxy activated Sepharose CL-6B, via coupling to a protein carrier. The choice of carrier protein is made such that the protein used for immunization would not be used, preventing the potential of isolating non-specific antibodies to the carrier protein.

Ensuing the binding, adsorbed phage is then separated by centrifugation followed by a series of wash steps removing the non-specific or weakly binding activities. The nature of the wash steps is such as to select for the type and nature of the interactions with the antigen of choice, i.e. selection of high salt washes would reduce the binding due to ionic interactions, or use of ethylene glycol would enable the reduction of hydrophobic interactions in favor of other binding affinities for example. An enhanced selection based on these wash conditions is not restricted to these broad based wash conditions but would also encompass the use of specific wash protocols based on the use of related antigens or substrates for the desired reaction. The elution of pools of phage is also based on the same set of criteria as used for the washes. The results of the combination of these approaches allowed selection of a vast matrix of related binding activities.

The desired pool(s) of binding activity is then amplified and subjected to detailed analysis of their binding and catalytic properties. The application of these types of selective washes and elutions enables the selection of desired properties. This need not be the final step in the process of mutagenesis and selection but is a stage on the route the desired structures with catalytic activity. Thus, this protocol would allow successive rounds of selections to mature the binding site.

The isolated potential candidate antibodies with or without catalytic activity are then introduced in the expression systems described above for the selection of activity based on the further selection directly for catalytic activity using antibiotic or auxotrophic selection (see Section B, Part 2). Also, these candidate molecules are selected for further rounds of mutagenesis and selection using this phage system. The technical details of this phage library approach are described in the publications by Cwirla, S., et al., PNAS, 87 (1990):6378-6387; and McCafferty, J., et al., Nature, 348 (1991):552-554 and Patent Application WO 92/01047.

Screening for Catalytic Antibodies

A. Selection of Antibodies for Beta- Lactamase Activity

Selection of catalytic activation of monolactam-based prodrugs can be done using antibodies produced by hybridomas or by mutating antibodies in *E. coli* to improve catalytic efficiency of existing antibodies.

1. Screening hybridoma-based antibodies for Beta-Lactamase Activity

In vitro detection of catalytic hydrolysis of monolactam prodrugs can be carried out with either hybridoma supernatant antibodies immobilized to plastic 96 well plates (by a method described below) or in solution with antibodies purified from ascites fluid.

Immobilization: Those hybridomas producing antibodies binding to hapten in an ELISA assay were selected for screening. Supernatants were pooled from exhausted 5 mL cultures, and the pH adjusted to 7-7.5 with 2N NaOH (20 μL). Cell debris was removed by centrifugation for 30 minutes at 2700 rpm, and supernatants (4 μL) were decanted into clean polypropylene tubes. Anti-mouse immunoglobulin affinity gel (Calbiochem, binding capacity 0.5-2 mg of immunoglobulin per mL of gel) was added as a 50% slurry in PBS (140 μL, containing 70 μL of gel) and the resulting suspensions were mixed gently for 16 hours at 25° C. A 96 well Millititer GV filtration plate (Millipore) was pre-wetted and washed in PBS containing 0.05% Tween-20. The affinity gel suspensions were spun in a centrifuge at 2500 rpm for 15 min, the bulk of the supernatant was removed, and the residual slurries (250 μL) from each polypropylene tube were each transferred to separate wells in the 96 well filter plate. Residual supernatant was removed by aspiration through the filter plate and the immobilized antibody was washed at 4° C with PBS/Tween (5 x 200 μL), PBS (3 x 200 μL), and 25 mM HEPES, pH 7.2 (3 x 200 μL).

Following appropriate incubation of antibody with prodrug, separation of drug from unhydrolyzed prodrug is accomplished by standard HPLC procedures. Hydrolysis of the prodrugs will result in liberation of an aromatic drug that can be easily detected by absorbance spectroscopy. Detection and quantitation of drug produced can be quantitated by an online spectral detector.

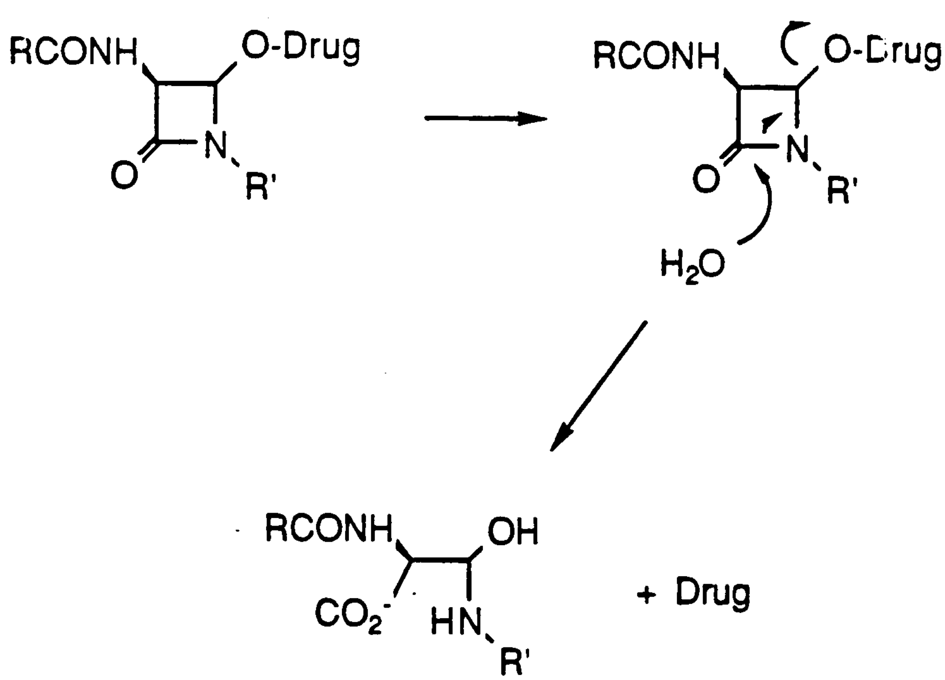
2. Screening Antibodies in *E. Coli* for Beta-Lactamase Activity

Efficiencies of catalytic antibodies are often substantially below those of natural enzymes. If current technologies are used to raise catalytic antibodies, many will be unsuitable for ffective commercial use without improvement by chemical or genetic alteration. Catalytic antibodies with β-lactamase activity will be particularly amenable for improvement by genetic mutation because their catalytic activity provides a rapid and convenient means by which host colonies of *E. coli* expressing antibody can be screened for activity. Because *E. coli*

(especially certain hypersensitive strains (Imada, A., et al., Nature 289 (1981):590-591; Dalbadie-McFarland, G., et al., Proc. Natl. Acad. Sci. USA 79 (1982):6409-6413) is killed by β-lactam antibiotics, a secreted antibody with β-lactamase activity will confer resistance to β-lactam toxicity. The more catalytically efficient the mutant antibody, the higher the minimum inhibitory concentration (MIC) of antibiotic for the host *E. coli*. Methods such as random mutagenesis of the genes for mildly catalytic antibodies will result in large numbers of *E. coli* colonies, creating large numbers of unique antibodies. Increased resistance to an appropriate β-lactam antibiotic will provide a rapid and efficient basis for screening enormous numbers of mutants and signal those antibodies with efficiencies above those of wild type antibodies.

Prodrug Strategy: Elimination of an Active Drug From the β-Lactam Ring

An active drug can be generated from an inactive prodrug as a consequence of hydrolysis of a substituted monocyclic β-lactam ring:



The substituents (R and R') will specifically depend on what is required to make the β-lactam an effective agent for disrupting the cell wall of a β-lactamase enzyme-deficient *E. coli* causing death or impaired growth. In addition, these substituents optionally are used in coupling a carrier protein (KLH or BSA) during immunization.

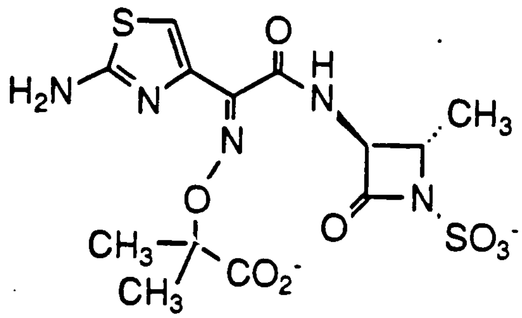
Cloning And Mutation Of Antibodies To Improve Catalytic Activity

Antibody genes producing catalytic antibodies will be cloned and expressed in *E. coli*. It will be critical to use a strain of *E. coli* that is hypersensitive to β-lactam antibiotics (i.e., one that lacks natural defenses against β-lactam antibiotics). Such strains exist that lack β-lactamase enzymes and/or penicillin binding proteins (Imada, A., et al., Nature 289 (1981):590-591; Dalbadie-McFarland, G., et al., Proc. Natl. Acad. Sci. USA 79 (1982):6409-6413). *E. coli* colonies will contain plasmid DNA encoding antibody genes mutated by either site-directed or random mutagenesis. The organisms will express and secrete altered antibody. Because many clones will be generated, each clone secreting antibodies of a different amino acid sequence, a rapid and labor-unintensive method of determining which mutants have increased catalytic activity will be used.

Screening of Mutant Catalytic Antibodies in *E. Coli*

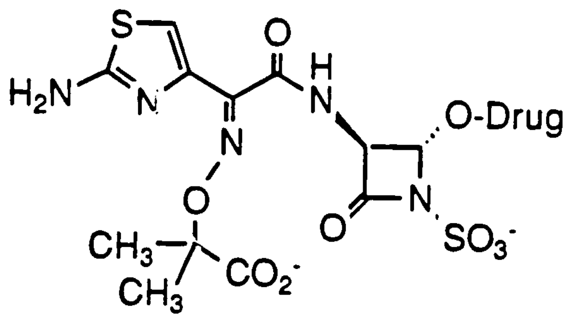
A sensitive and convenient method to screen *E. coli* mutants producing antibodies with β-lactamase activity is to detect the altered ability of the mutant to resist toxicity of a β-lactam antibiotic that resembles the prodrug. A preferred feature of this method is that the structures of the hapten, the prodrug, and the effective antibiotic used in screening all be similar enough to be recognized by the antibody. The hapten must elicit antibodies that not only bind and hydrolyze the prodrug but also an antibiotic (prodrug minus the drug) used to challenge the host organism, *E. coli*. An additional feature to be considered in the design of the prodrug is that upon hydrolysis it must expel the active drug. Based on these criteria, a number of different structures can be used for the prodrug as described elsewhere herein. One attractive example is to have a prodrug derivative of the monobactam antibiotic, aztreonam (Koster, W.

H., et al., Frontiers of Antibiotic Research, ed. H. Umezawa., (1987):211-226 Orlando, Academic Press).



Aztreonam

Aztreonam is an effective antibiotic against *E. coli* (MIC = 0.1 mg/mL) and is not degraded by human enzymes in the bloodstream. Haptens can be designed and prepared that hydrolyze the β-lactam ring of modified aztreonam to give elimination of an active drug.



Aztreonam-based Prodrug

Screening (in hypersensitive strains of *E. coli*) for efficient catalytic antibody-producing mutants is accomplished by challenging the host antibody-secreting colonies with aztreonam itself rather than with the actual prodrug. This is done because aztreonam (or a similar antibiotic) itself is an effective antibiotic against *E. coli* although it is not always clear what effect the addition of the drug (modified aztreonam) may have on aztreonam's antibiotic properties. The presence of the drug portion may abolish or diminish the antibiotic action of aztreonam on *E. coli*. Screening with aztreonam rather than with the larger aztreonam-drug conjugate is acceptable because the antibodies are raised to a hapten that included the drug or

an analog thereof and mutant antibodies will retain the capability to bind the drug. Screening is done by standard methods such as agar dilution (Sigal, I. S., et al., Natl. Acad. Sci. USA 79 (1982):7157-7160; Sowek, J. A., et al., Biochemistry 30 (1991):3179-88) or by using concentration gradients of aztreonam (Schultz, S. C., et al., J. Proteins 2 (1987):290-297).

Characterization Of Mutants

E. coli colonies found to be resistant to aztreonam are grown in larger quantities so that milligrams of antibody can be expressed and purified for further *in vitro* characterization. At this stage, antibodies will be purified and characterized in a buffered solution. A critical kinetic property is the ability to efficiently hydrolyze the β-lactam prodrug resulting in elimination of the active drug species. Lack of strong product inhibition by the prodrug (substrate), hydrolyzed aztreonam, or by the activated drug is required as well as efficient hydrolysis in human serum.

B. Isolation of Catalytic Antibodies that Activate Nucleoside Analog Prodrugs

Catalytic antibodies that activate nucleoside analog prodrugs can be isolated by either of two general principles; *in vivo* by selection methods or screening antibodies or phage-expressing antibodies by physicochemical methods (screening methods). The *in vivo* isolating method described below can be applied to screening antibodies for all of the nucleoside analog prodrugs. The screening methods are divided into two types based on the two kinds of inactivating groups claimed. One type of screening methods detects esterase activity and the other detects glycosidase activity. Screening can either be applied to antibodies purified from mouse ascites fluid, or at an earlier stage, to antibodies present in hybridoma supernatants. The methods listed here are specifically described for early screening of hybridoma supernatants for catalytic activity but can easily be adapted for the screening and assay of monoclonal antibodies purified from ascites.

1. Screening Of Catalytic Activation of Nucleoside Analog Prodrugs.

Screening is either carried out at an early stage in hybridoma supernatants using the immobilization procedures described in section A, or at a later stage using antibodies puried from mouse ascites.

Screening Antibodies For Galactosidase Catalytic Activity: To either antibody free in solution or antibody washed and immobilized, a solution of the prodrug in the appropriate assay buffer is added. Following incubation at 25° C for a time dependent on the uncatalyzed rate of prodrug activation, formation of activated drug is measured. The substrate solution is either removed from the well (in the immobilization method) to determine the extent of product formation or the product is measured *in situ* (as in the case of antibody free solution).

Detection of prodrug activation is carried out by colorometric or fluorometric determination of the generation of galactose, which accompanies prodrug activation.

One of a number of possible galactose detection methods is employed. Some sensitive and specific detection methods follow:

1. Radiolabelling of free galactose with ^{32}P-phosphate.
   a) Galactokinase (E.C. 2.7.1.6) is commercially available (Sigma Chemical Co., St. Louis, USA) and catalyzes the following reaction;

$$\text{galactose} + \text{ATP} \rightarrow \text{galactose-1-phosphate} + \text{ADP}$$

If the ATP (adenosine triphosphate) used has ^{32}P in the gamma phosphate position, free galactose generated by catalytic antibodies becomes radioactively-labelled. Labelled galactose-1-phosphate is separated from the other constituents in the reaction mixture by thin

layer chromatography (TLC) or high performance liquid chromatography (HPLC) and quantitated by scintillation counting.

2. Detection of catalysis using fluorescent or chromophoric aldehyde-reactive reagents.

In this type of detection method, galactose is non-catalytically reacted with commercially available (from, for example, Molecular Probes, Inc., Eugene, OR) aldehyde-reactive reagents to yield a colored or fluorescent derivative. The product of the reaction with galactose is isolated by HPLC or by TLC and detected by absorbance or by fluorescence by standard means.

One potential reagent is dansyl hydrazine (Molecular Probes, Inc.). Dansyl hydrazine reacts under mild conditions with aldehydes to give a fluorescent product (Eggert, F. M., et al., J. Chromatogr. 333 (1985):123; Avigad, G., J. Chromatogr. 139 (1977):343) that is detectable at low concentrations upon TLC or HPLC of the reaction mixture. Other potential reagents that are more useful than dansyl hydrazine because of possible lower detection limits, greater reaction specificity, or milder reaction conditions are other fluorescent hydrazides that are commercially available such as coumarin hydrazide, fluorescein thiosemicarbazide (Molecular Probes, Inc.). These reagents are compared to see which best suits the specific requirements.

3. Detection of galactose with color-generating specific enzymes.

a) One enzyme that can be used to detect galactose is galactose dehydrogenase (E.C. 1.1.1.48) (Sigma Chemical Company, St. Louis, MO, USA) which catalyzes the following oxidation-reduction reaction;

$$\text{galactose} + \text{NAD+ (no color)} \longrightarrow \text{galactonate} + \text{NADH (color)} + \text{H+}$$

The oxidation of galactose is accompanied by the reduction of nicotinamide adenine dinucleotide (NAD^+). The reduced form of NAD^+, NADH, is colored and its appearance is monitored spectrophotometrically at 340 nm.

b) An alternative enzyme that is useful in detecting galactose is galactose oxidase, (E.C. 1.1.3.9) which, used in combination with peroxidase and o-tolidine, will cause a color change in response to the presence of free galactose generated by a catalytic antibody. The coupled reactions are as follows. The first reaction is catalyzed by galactose oxidase and the second by peroxidase, both available from Sigma Chem. Company;

1. galactose + $O_2 \rightleftharpoons$ galactonate + H_2O_2
2. H_2O_2 + o-tolidine $\rightleftharpoons$ H_2O + "colored product"

The colored product generated can be measured spectrophotometrically.

<u>Screening Antibodies For Esterase Catalytic Activity:</u> To immobilized washed antibody or antibody free in solution, a solution of the prodrug (unless otherwise indicated) in the appropriate assay buffer is added. Following incubation at a suitable temperature such as 25° C for a time dependent on the uncatalyzed rate of prodrug activation, formation of activated drug is measured as described.

Detection of prodrug formation can be detected by pH change that accompanies ester hydrolysis in weakly buffered solutions. Changes in pH can be detected by including an acid-base indicator in the solution, such as phenol red (Benkovic, P. A., et al., <u>Biochemistry</u> 18 (1979):830), which changes color with pH change. Alternatively, a method that is more sensitive is to use a pH stat or pH meter equipped with a fine-tipped electrode that can be inserted into the wells (Lazar Research Laboratories, Los Angeles, CA) to measure pH

changes. For screens involving measuring changes in pH, it may be necessary during the incubation to keep the wells under nitrogen or argon gas to prevent pH changes from atmospheric carbon dioxide.

Hydrolysis of aromatic ester-protected prodrugs results in the liberation of an acidic aromatic group which can easily be separated by conventional chromatographic means on an HPLC (anion exchange or reverse phase columns). Furthermore, detection of the aromatic ring eluting from the HPLC can be easily accomplished using an online UV absorbance detector.

A third method for *in vitro* detection of hydrolysis of aromatic ester nucleoside analogs is to use an enzyme-linked assay. One inexpensive commercially-available enzyme (Sigma Chemical Company, St. Louis, MO) that could be used for this purpose is thymidine phosphorylase (E.C. 2.4.2.4). This enzyme converts the substrates thymidine and orthophosphate to the products thymine and 2-deoxy-D-ribose-1-phosphate. Rather than the prodrug being screened here, a conjugate of the inactivating ester with thymidine will be used (the same types of compounds that will be used in biological screening with auxotrophic bacterial mutants). This enzyme will not catalyze the phosphorylation of the aromatic ester protected thymidine, but only free thymidine produced by the catalytic antibody. To the wells will be added the thymidine phosphorylase, the thymidine version of the prodrug, and ^{32}P-labelled orthophosphate. After incubation of the buffered components with the immobilized antibodies, aliquots are run on TLC to separate radiolabelled orthophosphate and 2-deoxy-D-ribose-1-phosphate. The ^{32}P can then be detected on the TLC plates by autoradiography.

2. <u>Thymidine Auxotrophic Selection for Isolation of Catalytic Antibodies with Esterase Activity for Nucleoside Analogue Prodrugs</u>

Bacterial expression of antibodies promises to provide large numbers of different antibodies to screen for catalytic activity. However, the usefulness of this methodology is dependent on the availability of effective methods of selecting those colonies producing active antibody. A

powerful approach is to use biological selection, in which only those colonies producing catalytic antibody are able to survive. One way in which this selection can be carried out is for the catalytic antibody to supply a particular nutrient in which the bacteria are deficient; survival is dependent on the antibody cleaving a substrate which releases the required nutrient. This type of selection to obtain prodrug-cleaving catalytic antibodies, is described below.

To produce a catalytic antibody capable of cleaving a prodrug, thereby releasing a nucleoside analogue (e.g., fluorouridine, fluorodeoxyuridine, fluorouridine arabinoside, cytosine arabinoside, adenine arabinoside, guanine arabinoside, hypoxanthine arabinoside, 6-mercaptopurineriboside, theoguanosine riboside, nebularine, 5-iodouridine, 5-iododeoxyuridine, 5-bromodeoxyuridine, 5-vinyldeoxyuridine, 9-[(2-hydroxy)ethoxy]methylguanine (acyclovir), 9-[(2-hydroxy-1-hydroxymethyl)-ethoxy]methylguanine (DHPG), azauridien, azacytidine, azidothymidine, dideoxyadenosine, dideoxycytidine, dideoxyinosine, dideoxyguanosine, dideoxythymidine, 3'-deoxyadenosine, 3'-deoxycytidine, 3'-deoxyinosine, 3'-deoxyguanosine, 3'-deoxythymidine), prodrug activating antibodies are produced by bacterial expression, and those able to supply thymidine to otherwise thymidine-deficient bacteria are selected. Thymidine bears a close structural resemblance to fluorouridine and the other nucleoside analogues listed above; therefore, a catalytic antibody able to release fluorouridine (or any of the other nucleoside analogues listed above) from a prodrug is able to release thymidine from the equivalent substrate in which fluorouridine (or any other nucleoside analogue of interest) has been replaced by thymidine. This is illustrated below for a fluorouridine-based prodrug. Thymidine-deficient bacteria are applied with substrate thymidine derivatized by the same promoiety as the fluorouridine prodrug; colonies producing a catalytic antibody able to cleave the pronutrient can utilize released thymidine and therefore survive. Antibody from these surviving colonies is then screened for cleavage of the prodrug to give fluorouridine.

Blocking thymidine production is a potent method of arresting bacterial cell growth. Thymidine is essential for DNA synthesis, and it is obtained only by enzymatic methylation of deoxyuridine. As the base thymine is not found in RNA, there is no possibility of supplementing the thymidine pool by degradation of RNA blocking the conversion of deoxyuridine to thymidine rapidly shuts down DNA synthesis. Therefore, one way of blocking thymidine synthesis is to inhibit the enzymes thymidylate synthetase or dihydrofolate reductase (DHFR). Fluorodeoxyuridylate is an irreversible inhibitor of thymidylate synthetase, but it also gives rise to synthesis of defective RNA, so that antibody-mediated release of thymidine may not be sufficient to prevent cell death. Methotrexate is a highly specific inhibitor of DHFR; however, tetrahydrofolate, the product of the enzymatic reduction, is also required for the biosynthesis of purines and certain amino acids. Nevertheless, the purine pool is maintained by supplementing the growth medium with hypoxanthine so that the methotrexate-treated bacteria would then have a unique requirement for thymidine. (Another folate analogue, trimethoprim, is an even more potent inhibitor of bacterial DHFR than methotrexate, and is used if necessary; Gilman, A. G., et al., The Pharmacological Basis of Therapeutics (1985):1263-1268).

An alternative way of selecting for cleavage of thymidine-based prodrug is to use a strain of *E. Coli* deficient in thymidylate synthetase (Neihardt, F. C., Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology (1987). Use of a strain in which expression of the enzyme is temperature sensitive allows all the colonies initially to be grown with the enzyme fully expressed. Raising the temperature then shuts down enzyme expression, and only those colonies producing an antibody able to cleave the thymidine-based prodrug are able to survive.

C. Screening Of Catalytic Activation Of Cyclophosphamide Prodrug

Immobilization and Screening of Catalytic Monoclonal Antibodies

Immobilization: Immobilization is carried out as described in Section A. Alternatively, screening is carried out with antibody free in solution.

Screening Antibodies for Catalytic Activity: To antibody in solution or immobilized washed antibody, a solution of the prodrug in the appropriate assay buffer is added. Following incubation at 25° C for a time dependent on the uncatalyzed rate of prodrug activation, formation of activated drug is measured. The substrate solution is either removed from the solution to determine the extent of product formation or the product is measured *in situ*.

Detection of prodrug activation is carried out by colorometric or fluorometric determination of a byproduct that accompanies prodrug activation--acrolein.

One of a number of possible acrolein detection methods is employed. Some potentially sensitive and specific detection methods follow:

1. Detection of acrolein using enzymes that catalyze reactions of acrolein.
   a) One enzyme that can be used to detect acrolein formation is alcohol dehydrogenase (E.C. 1.1.1.1) Alcohol dehydrogenase is commercially available (Sigma Chemical Company) and catalyzes the following reaction (where, for example, the aldehyde is acetaldehyde and the alcohol is ethanol);

$$\text{aldehyde} + \text{NADH (colored)} + \text{H+} \rightleftharpoons \text{alcohol} + \text{NAD+ (no color)}$$

The oxidation of NADH to NAD^+ is accompanied by a color change centered at 340 nm. This color change is a commonly used with this enzyme to monitor its activity. The compound, acrolein, will be accepted as the aldehyde substrate by alcohol dehydrogenase since it closely resembles acetaldehyde, and the enzyme is not particularly strict with the exact structure of its substrates. There are different types of alcohol dehydrogenase commercially

available from different species (yeast and equine, for example) and the enzymes from different species differ somewhat in their substrate specifities so that if the enzyme from one species does not oxidize acrolein, another may.

b) The reaction catalyzed by aldehyde dehydrogenase (E.C. 1.12.1.5), also commercially available from Sigma Chemical Company, is similar in that aldehyde substrates are accepted and a color change occurs with the reaction. In this reaction, the aldehyde is oxidized to a carboxylic acid (acetaldehyde to acetic acid, for example);

$$\text{aldehyde} + \text{NAD}^+ (\text{no color}) \rightleftharpoons \text{acid} + \text{NADH (color)} + \text{H}^+$$

In this case a disappearance of color at 340 nm will accompany the transformation of substrate since NAD^+ is converted to NADH, rather than the other way around as with alcohol dehydrogenase.

c) A third possible enzyme-coupled detection method employs both alcohol oxidase (E.C. 1.1.3.13) and peroxidase (E.C. 1.11.1.7). Alcohol oxidase can convert an aldehyde to a carboxylic acid using molecular oxygen and creating hydrogen peroxide;

$$\text{aldehyde} + O_2 \rightleftharpoons \text{acid} + H_2O_2$$

Alcohol oxidase is commercially available (Sigma Chemical Company) and on the basis of published literature will accept acrolein as a substrate (Guibault, G. G., Handbook Of Enzymatic Methods Of Analysis (1976):244-248, New York: Marcel Dekker). The formation of hydrogen peroxide by alcohol oxidase is monitored by adding peroxidase (Sigma Chemical

Company) to the reaction mixture along with the chromophoric peroxidase substrate, o-dianisidine. Peroxidase will catalyze the following reaction;

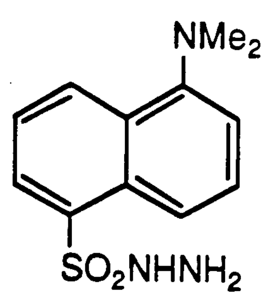
$$H_2O_2 + \text{o-dianisidine} \rightleftharpoons H_2O + \text{"colored product"}$$

The colored product is spectrophotometrically observable at 456 nm.

2. Detection of catalysis using fluorescent or chromophoric aldehyde-reactive reagents.

In this type of detection method, acrolein is non-catalytically reacted with commercially available aldehyde-reactive reagents (from, for example, Molecular Probes, Inc., Eugene, OR, USA) to yield a colored or fluorescent derivative. The product of the reaction with acrolein is isolated by high performance liquid chromatography (HPLC) or by thin layer chromatography (TLC) and detected by absorbance or by fluorescence by standard means.

One potential reagent is dansyl hydrazine (Molecular Probes, Inc.).



Dansyl hydrazine reacts under mild conditions with aldehydes to give a fluorescent product (Eggert, F. M., et al., J. Chromatogr. 333 (1985):123; Avigad, G., J. Chromatogr. 139 (1977):343) that is detectable at low concentrations upon TLC or HPLC of the reaction mixture.

Other reagents that are more useful than dansyl hydrazine because of lower detection limits, greater reaction specificity, or milder reaction conditions are other fluorescent hydrazides that

are commercially available such as coumarin hydrazide, fluorescein thiosemicarbazide (Molecular Probes, Inc.). These reagents are compared to see which best suits the specific requirements.

D. Screening for Antibody Catalyzed Liberation of Doxorubicin from Prodrugs

1. Background. Doxorubicin prodrug activation can be detected in either of two basic ways; *in vitro* detection by observing the inherent physical changes that accompany the chemical transformation of prodrug to active drug, or *in vivo* detection by biological screening for the toxic effects of the activated drug.

2. Screening. Screening of antibodies in monoclonal cell line supernatants using the immobization method described in Section A or of antibodies purified from ascites is done by standard methods of either thin layer chromatography (TLC) or high performance liquid chromatography (HPLC). Typically, the reaction mixture contains 200 micromolar prodrug, approximately 1 micromolar antibody, 140 mM sodium chloride, and is buffered at pH 7.4 in 10 mM HEPES buffer. Changes in component concentrations and in pH are also tested. Typical alternative pH values are pH 5.0 in which MES buffer replaces HEPES, and pH 9.0 in which Tris buffer replaces HEPES. The temperature is typically at 25° C but is raised if the background (uncatalyzed) hydrolysis of the prodrug is not dramatically increased at higher temperatures.

Doxorubicin, its prodrug forms, and the cleaved inactivating pro moiety can all be detected by absorbance or fluorescence. Doxorubicin, and presumably the doxorubicin prodrug both absorb strongly in ultraviolet and visible light (Absorption max (methanol): 233, 252, 288, 479, 496, 529 nm). The aromatic inactivating pro moiety absorbs strongly in the ultraviolet at 260-280 nm as well as 220 nm.

Observation of antibody-catalyzed prodrug activation by TLC is carried out with either purified antibodies or, using the 96-well plate early screening detection method described herein, with impure antibodies in cell culture supernant. TLC of doxorubicin prodrug activation is carried out by standard methods resulting from separation of drug and prodrug on the TLC plate. When the doxorubicin prodrug is hydrolyzed to form free doxorubicin, a primary amino group is exposed on the drug. With proper choice of TLC matrix and solvent systems, separation of pro form from active drug is readily accomplished. Detection of TLC-separated drug and prodrug is either visible inspection of orange-red color or by the natural fluorescence of doxorubicin using an ultraviolet-emitting light. Also, when prodrug activation occurs, a free carboxyl group is formed in the leaving aromatic pro moiety which gives this newly formed compound properties that allow separation by TLC from both prodrug and doxrubicin.

Screening of active drug formation is also carried out by HPLC under standard conditions. Visible and ultraviolet detection of prodrug depletion or drug or pro moiety formation is used with an on-line absorbance or fluorescence detector. Prodrug, drug, and liberated pro moiety is separated on a reverse phase column using common solvent systems which is optimized for best separation. Conditions that are optimized are; type of reverse phase column, solvent flow rate, solvent mixture components, and elution profile (isocratic elution or gradient elution).

3. Selection. Doxorubicin is a general cytotoxin that is toxic to both bacterial and mammalian cells. Screening for the biological effects of antibody-liberated doxorubicin permits identification of cell lines (bacterial or hybridoma) producing large amounts of catalytically active prodrug-activating antibody. If the prodrug is not cytotoxic, only those cell lines producing prodrug-activating antibody are killed by the prodrug. This idea is analogous to that delineated herein for biological selection of cell lines by screening for increased resistance to β-lactam antibiotics and by ability of catalytic antibody cell lines

deficient in thymidine synthetase to produce thymidine by prodrug cleavage. In the case of doxorubicin prodrugs, screening differs in that selection is for cell death by suicide caused by prodrug activation (rather than for catalytic antibody-conferred enhanced survival abilities). Thus, in the case of biological screening for doxorubicin production, an aliquot of each cell line is kept aside and not used in the screening so that the catalytic antibody producing cell lines is not lost during selection. In practice, a series of colonies of monoclonal cells (hybridoma or bacterial) producing antibody are exposed to serial dilutions of the prodrug. Those cell lines that show increased susceptibility to death in a dose-dependent manner are studied further; those antibodies are isolated and further characterized in a pure state. Alternatively, instead of serial dilutions of prodrug administered to a series of colonies of the same cell line, a single dose of prodrug is administered in a concentration calculated to bring about death by an arbitrarily-decided minimally satisfactory kinetic rate of antibody catalysis in the time of the experiment.

E. Screening Of Antibodies For Catalytic Activation Of Melphalan Prodrugs

Antibodies are either screened at an early stage in hybridoma supernatants by the 96 well plate immobilization technique (described in Section A) or at later stage from mouse ascites. In either case catalysis can be detected by normal methods of HPLC separation of substrates and products. The substrate (prodrug) and products (drug and pro moiety) are all aromatic and can be detected at low levels using a UV detector online with the HPLC apparatus. In the case of the early screen, aliquots from the wells following a suitable incubation time with antibody are withdrawn and injected into the HPLC. Likewise with antibody from ascites, reaction aliquots are injected onto the HPLC and separation of substrate and products as well as detection and quantitation are carried out.

Formulation and Administration

The present invention also encompasses pharmaceutical compositions, combinations and methods for treating cancers and other tumors. More particularly, the invention includes

combinations comprising immunoconjugates (targeting protein and catalytic protein, or targeting antibody and catalytic antibody (bispecific antibodies) and the corresponding prodrug or prodrugs for use in a method for treating tumors wherein a mamalian host is treated in a pharmaceutically acceptable manner with a pharmaceutically effective amount of a targeting protein catalytic protein conjugate or conjugates or bispecific antibody or antibodies and a pharmaceutically effective amount of a prodrug or prodrugs. The combination and methods of this invention are useful in treating humans and animals.

In an advantageous embodiment, the immunoconjugate is administered prior to the introduction of the prodrug into the host. Sufficient time is then allowed between administration of the immunoconjugate and the prodrug to allow the targeting protein of the immunoconjugate to target and localize at the tumor site. Such sufficient time may range from 4 hours to one week depending upon the conjugate used. The period of time between the end of administration of the immunoconjugate and the beginning of administration of prodrug varies depending on the site to be targeted and the nature of the immunoconjugate and prodrug, together with other factors such as the age and condition of patient. More than one administration of prodrug may be necessary to achieve the desired therapeutic effect. Thus, the exact regime will usually need to be determined empirically, with the aim of achieving a maximal concentration of immunoconjugate at the target site and a minimal concentration elsewhere in patient, before the prodrug is administered. In this way, an optimum selective therapeutic effect can be achieved.

The immunoconjugate is administered by any suitable route, preferably parenterally, e.g., by injection or infusion. These compounds are administered using conventional modes of administration including, but not limited to, intravenous, intraperitioneal, oral, intralymphatic, or administration directly into the tumor. Intravenous administration is particularly advantageous.

The compositions of the invention--comprising the immunoconjugates or prodrugs--may be in a variety of dosage forms which include, but are not limited to, liquid solutions or suspensions, tablets, pills, powders, suppositories, polymeric microcapsules or microvesicles, liposomes, and injectable or infusible solutions. The preferred form depends upon the mode of administration and the therapeutic application. For example, oral administration of the antibody-enzyme conjugate or bispecific antibody may be disfavored because the conjugate proteins tend to be degraded in the stomach if taken orally, e.g., in tablet form.

Suitable formulations of the immunoconjugate or prodrug for parenteral administration include suspensions, solutions or emulsions of each component in oily or aqueous vehicles and optionally contain formulatory agents such as suspending, establishing and/or dispersing agents. Alternatively, the immunoconjugate or prodrug is in powder form for reconstituting with a suitable vehicle, e.g., sterile pyrogen-free water before use. If desired, the immunoconjugate antibody and/or prodrug is presented in unit dosage form. Formulations are conveniently prepared in isotonic saline for injection.

The most effective mode of administration and dosage regimen for the compositions of this invention depends upon the severity and course of the disease, the patient's health and response to treatment and the judgement of the treating physician. Accordingly, the dosages of the immunoconjugates and prodrugs should be titrated to the individual patient.

Nevertheless, an effective dose of the immunoconjugate of this invention is in the range of from about 1.0 to about 100 mg/m^2. An effective dose of the prodrug of the invention will depend upon the particular prodrug used and the parent drug from which it is derived. The precise doses at which the immunoconjugate and prodrug will be administered will depend on the route of administration, body weight, and pathology of the patient, the nature of the prodrug, and the catalytic properties of the immunoconjugate. Since the prodrug is less

cytotoxic than the parent drug, dosages in excess of those recognized in the art for the parent drug may be used.

The prodrug is administered at doses in general use for the administration of the drug itself but will preferably be administered at lower doses, for example, or around 0.001 to 0.5 times the normally administered dose of drug alone.

Another embodiment of this invention of this invention provides a method of combination chemotherapy using several prodrugs and only a single antibody-enzyme conjugate. According to this embodiment, a number of prodrugs are used that are all substrates for the same enzyme or catalytic antibody in an immunoconjugate. Thus, a particular antibody-enzyme conjugate or bispecific antibody converts a number of prodrugs into cytotoxic form, resulting in increased antitumor activity at the tumor site.

Still another embodiment of this invention involves the use of a number of immunoconjugates wherein the specificity of the antibody varies, i.e., a number of immunconjugates are used, each one having an antibody that binds specifically to a different antigen on the tumor of interest. The enzyme component of these immunoconjugates is the same or may vary. This embodiment is especially useful in situations where the amounts of the various antigens on the surface of a tumor is unknown and one wants to be certain that sufficient enzyme is targeted to the tumor site. The use of a number of conjugates bearing different antigenic specificities for the tumor increases the likelihood of obtaining sufficient enzyme at the tumor site for conversion of a prodrug or series of prodrugs. Additionally, this embodiment is important for achieving a high degree of specificity for the tumor because the likelihood that normal tissue will possess all of the same tumor-associated antigens is small [cf., I. Hellstrom, et al., "Monoclonal Antibodies To Two Determinants Of Melanoma-Antigen p97 Act Synergistically In Complement-Dependent Cytotoxicity", J. Immunol. 127 (No. 1), (1981):157-160].

In some patients with multiple metastatic lesions, tumor imaging has proven difficult due to the heterogeneity of the tumor cells wherein only some of the cells express the targeted antigens. In such tumors, where intra or inter-tumor heterogeneity is known to exist, a cocktail of monoclonal antibodies recognizing different tumor antigens are used to activate the prodrug. This approach offers the potential of achieving a higher total concentration of drug at the tumor site in the cases where antigen heterogeneity exists (Wahl, R., Cancer Research, Suppl., (1990):941s-948s).

The following examples are illustrative, but not limiting of the methods and compositions of the present invention. Other suitable modifications and adaptations of a variety of conditions and parameters normally encountered in clinical therapy which are obvious to those skilled in the art are within the spirit and scope of this invention.

Examples

Example 1a: Preparation of the Prodrugs, linear trimethylbenzoyl, trimethoxybenzoyl-, trimethoxybenzoyl-, and 5'-0-(2,6-dimethoxybenzoyl)-5-fluorouridine, Compounds 1a, 1b, and 1c.

5'-O-(2,4,6-Trimethylbenzoyl)-5-fluorouridine **1a**, 5'-O-(3,4,5-Trimethoxybenzoyl)-5-fluorouridine **1b**, and 5'-0-(2,6dimethylbenzoyl)-5-fluorouridine **1c**. (For individual reference, compound numbers in bold in the following text refer to the compounds in the synthetic schemes shown in the figures.) Refer to Figures 1a and 1c for the bold numbered compounds in this Example.

The preparation of 5'-O-(2,4,6-trimethylbenzoyl)-5-fluorouridine **1a** and 5'-O-(3,4,5-trimethoxybenzoyl)-5-fluorouridine **1b** was achieved with the reaction of 2,4,6-

trimethylbenzoyl-chloride and 3,4,5-trimethoxybenzoyl chloride with 2',3'-O-isopropylidene-5-fluorouridine **65** (prepared in Example 16) in pyridine followed by acid hydrolysis with 50% formic acid at 65° C.

The preparation of 5'-O- (2,6 dimethoxybenzoyl)-5-fluorouridine **1c** was achieved by reaction of 2,6 dimethoxybenzoyl chloride and compound **65** in pyridine followed by acid hydrolysis using 50% formic acid at 65° C.

In detail, the synthesis is as follows:

5'-0-(2,4,6-Trimethylbenzoyl)-5-fluorouridine **1a**

A mixture of 328 mg of 2,4,6-trimethylbenzoic acid and 3 mL of thionyl chloride was stirred at room temperature for 2 hours. The volatile components were evaporate *in vacuo*, the residue was redissolved in 5 mL of CH_2Cl_2, and the volatile components were again evaporated *in vacuo* to give 2,4,6-trimethylbenzoyl chloride.

Anhydrous pyridine (10 mL) was evaporated three times from 151 mg of 2',3'-0-isopropylidene-5-flurorouridine, Compound **65** of Example 16. Pyridine (1 mL) was added to the residue, the mixture was cooled by an ice bath, and a solution of 456 mg of 2,4,6-trimethylbenzoyl chloride in 4 mL of CH_2Cl_2 was added dropwise. One hour after the completion of addition, 1 mL of MeOH was added. After standing for 16 hours, the volatile components were evaporated *in vacuo*, the residue was dissolved in ethyl acetate (75 mL) and washed with saturated $NaHCO_3$ (2 x 50 mL) and water (25 mL), dried over anhydrous $MgSO_4$, concentrated *in vacuo*, and purified by flash chromatography (50% ethyl acetate/hexane, R_f 0.63) to give 142 mg of the product as a colorless solid, ^1H NMR (DMSO-d_6) δ 1.27 (s, 3), 1.48 (s, 3), 2.18 (s, 6), 2.22 (s, 3), 4.30 (m, 1), 4.45 (m, 2), 4.81 (m, 1), 5.10 (dd, 1), 5.78 (d, 1), 6.87 (s, 2), 8.05 (d, 1), 11.97 (d, 1).

A mixture of 440 mg of the above acetonide in 6 mL of 50% formic acid was heated at 65° C for 2 hours. The volatile components were evaporated *in vauco*. The residue (408 mg) had: ^{1}H NMR (DMSO-d6) δ 2.13 (s, 6), 2.19 (s, 3), 3.92 (m, 1), 4.05 (m, 2), 4.44 (m, 2), 5.72 (d, 1), 6.83 (s, 2), 7.81 (d, 1), 11.82 (bs, 1).

The residue was purified by reverse phase HPLC on a C18 column eluted with 40% CH_3CN/H_2O to give 260 mg of the product, Compound **1a**, as a colorless solid.

5'-O-(3,4,5-Trimethoxybenzoyl)-5-fluorouridine **1b**

0.604 g of 2',3'-isopropylidene-5-fluorouridine, Compound **65** of Example 16, was, after azeotropic removal of moisture from pyridine, dissolved in 4 mL of dry pyridine and cooled to 0° C. A solution of 0.92 g of 3,4,5-trimethoxybenzoyl chloride in 4 mL of dichloromethane was added dropwise over 1 hour period at 0° C. After stirring for a further 1 hour at 0° C, the resulting mixture was quenched by the addition of 7.5 mL of methanol. The mixture was evaporated to a syrup, redissolved in ethyl acetate (75 mL) and washed with saturated sodium hydrogen carbonate (2 x 75 mL) and water (50 mL). The crude mixture was then purified by flash chromatography using ethyl acetate/ hexane to give 0.30 g of 5'-O-(3,4,5-trimethoxybenzoyl)-2',3'-isopropylidene-5-fluorouridine: ^{1}H NMR (DMSO-d_6) δ 1.32 (s, 3), 1.52 (s, 3), 3.73 (s, 3), 3.85 (s, 6), 4.40 (m, 1), 4.53 (m, 2), 4.94 (m, 1), 5.09 (m, 1), 5.77 (d, 1), 7.22 (s, 2), 8.01 (d, 1), 11.90 d, 1).

0.30 g of 5'-O-(3,4,5-trimethoxybenzoyl)-2',3'-isopropylidene-5-fluorouridine was dissolved in 4.2 mL of 50% aqueous formic acid and was heated with stirring at 65° C for 2 hours. The mixture was concentrated *in vacuo* and was then purified by flash chromatography using ethyl acetate to give 0.15 g of 5'-O-(3,4,5-trimethoxybenzoyl)-5-

fluorouridine **1b**: ^1H NMR (CD_3CN) δ 3.81 (s, 3), 3.86 (s, 6), 4.15-4.28 (m, 3), 4.53 (dd, 1), 4.63 (dd, 1), 5.75 (d, 1), 7.30 (s, 2), 7.59 (d, 1)

5'-O-(2,6 dimethoxybenzoyl)-5-fluorouridine, **1c**: To a solution of compound **65** (0.45 g, 1.5 mmol) in pyridine (3 mL) at 0° C under an argon atmosphere, a solution of 2,6 dimethoxybenzoyl chloride (0.4 g, 4 mmol) in methylene chloride (2 mL) was added dropwise through a syringe and the resulting mixture was stirred at that temperature for 4 hours. After completion of the reaction, methanol (3 mL) was added to the reaction mixture and solvents were removed *in vacuo* . The resulting material was dissolved in ethyl acetate (75 mL) and washed with a saturated solution of sodium bicarbonate (2 x 20 mL) in water (20 mL). The organic layer was separated, dried, concentrated, and flash chromatoghaphed to afford the coupled compound as an oily material (0.66 g, 95 %, Rf, 0.46, silica, methylene chloride, methanol, hexane, 80, 1, 19).

^{1}H NMR (DMSO-d_6): 8.98 (bs, 1H), 7.56 (d, 1H), 7.32 (m, 1H), 6.56 (d, 2H), 5.92 (m, 1H), 4.82 (m, 2H), 4.72 (m, 1H), 4.62 (m, 2H), 4.40 (m, 1H), 3.80 (s, 6H), 1.61 (s, 3H), 1.40 (s, 3H).

A solution of above compound (0.47g, 1 mmol) in formic acid (50%, 6 mL) was heated at 65° C with stirring under an argon atmosphere for 2 hours. After completion of the reaction, solvent was removed *in vacuo* and the resulting material was purified by reverse phase HPLC to afford the compound **1c** (0.27 g, 65%).

^{1}H NMR: 7.82 (d, 1H), 7.38 (1, 1H), 6.62 (d, 2H), 5.80 (d, 1H), 4.52 (dd, 2H), 4.16 (m, 3H), 3.86 (s, 6H).

Example 1b: Preparation f the hapten for Prodrug 1b in Example 1a, the linear phosphonate of trimethoxybenzoate-5-fluorouridine, Compound 4.

Refer to Figure 1b for the bold numbered compounds in this Example.

Uridine was iodinated at the 5 position to give iodide **3a** (Robins, J. M., et al., Can. J. Chem. 60 (1982):554-557). The hydroxyl groups were protected to give iodide **3c**. 3-Butyne-1-ol was transformed in four steps to alkyne **3d**. Alkyne **3d** and iodide **3c** are coupled using a Pd(II) catalyst to give nucleoside analog **3e** (Robins, J. J., et al., J. Org. Chem. 48 (1983):1854-1862). Selective deprotection gives alcohol **3f**.

Dibenzyl 3,4,5-trimethoxyphenylphosphonate **2** can be prepared from the reaction of 3,4,5-trimethoxybromobenzene with dibenzyl phosphite at high temperature in the presence of tetrakis(triphenylphosphine)palladium (0), triethylamine and toluene following the procedure of J. Med. Chem. 32 (1989):1580-1590. Reaction of diester **2** with 1 equivalent of PCl_5 gives monochloridate **2a**, which is reacted with alcohol **3f** to give diester **3g**. Reduction and basic hydrolysis gives hapten **4**, which can be linked to a carrier protein via the primary amino group.

In detail, the synthesis is as follows:

5-Iodouridine **3a**

5-Iodouridine was prepared following the procedure of Robin, M. J., et al., Can J. Chem. 60 (1980):554-557, incorporated herein by reference.

5'-0-*tert*-Butyldimethylsilyl-5-iodouridine **3b**

Imidazole (216 mg) was added to a mixture of triol **3a** (490 mg) and *tert*-butyldimethylchlorosilane (239 mg) in 1 mL of DMF cooled by an ice bath. The mixture was

allowed to warm to room temperature. After 16 hours, the mixture was poured into 0.1 M HCl (25 mL) and extracted with ethyl acetate (3 x 50 mL), the aqueous phases were washed with water, dried over anhydrous $MgSO_4$, and concentrated *in vacuo*. Purification by flash chromatography (7% MeOH/CH_2Cl_2) gave 460 mg of the product as a colorless solid: ^1H NMR (DMSO-d_6) δ 0.08-0.12 (m, 6), 0.90 (s, 9), 3.72 (dd, 1), 3.80 (dd, 1), 3.90 (bs, 2), 4.02-3.98 (m, 1), 5.76 (d, 1), 7.93 (s, 1), 11.74 (bs, 1).

5'-0-*tert*-Butyldimethylsilyl-2',3'-0-3-N-tris(4-methylbenzoyl)-5-iodouridine **3c**

Anhydrous pyridine (3 x 15 mL) was evaporated *in vacuo* from 450 mg of diol **3b**. The residue was dissolved in 15 mL of pyridine, and 650 µL of triethylamine followed by 612 µL of 4-toluoyl chloride were added. The mixture was heated at 50° C for 5 hours. The mixture was cooled to room temperature, and an additional 410 µL of triethylamine and 390 mL of 4-toluoyl chloride were added. Heating was continued for 16 hours, and then the volatile components were evaporated *in vacuo*. The residue was dissolved in chloroform (50 mL), washed with 1M HCl (3 x 50 mL) and water (2 x 50 mL), concentrated *in vacuo*, and purified by flash chromatography (30% ethyl acetate/hexane) to give 534 mg of the product as a colorless solid.

^1H NMR ($CDCl_3$) δ 0.33 (s, 6), 1.07 (s, 9), 2.35 (s, 3), 2.38 (s, 3), 2.41 (s, 3), 4.06 (bs, 2), 4.47 (bs, 1), 5.58 (dd, 1), 5.73 (d, 1), 6.57 (d, 1), 7.13 (d, 2), 7.15-7.25 (m, 4), 7.79 (d, 4), 7.90 (d, 2), 8.34 (s, 1).

4-(4-Toluenesulfonyloxy)-1-butyne

Triethylamine (12.2 mL) was added dropwise to a mixture of 3-butyn-1-ol (5.09 g) and 4-toluenesulfonyl chloride (16.95 g) in 50 mL of CH_2Cl_2 cooled by an ice bath. The mixture was allowed to warm to room temperature. After 21 hours, the mixture was poured into ethyl

acetate (150 mL) and washed with 0.1M HCl (75 mL), saturated $NaHCO_3$ (75 mL), and brine (75 mL) and the organic phase was dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. Purification by flash chromatography (10% ethyl acetate/hexane) gave 16.57 g of the product as a colorless solid.

IR (neat) 3293, 3067, 2963, 2925, 2125, 1734, 1599, 1496, 1465, 1360, 1308, 1293, 1246, 1190, 1176, 1098, 1021, 982, 906, 817, 769, 665 cm^{-1}, ^1H NMR ($CDCl_3$) δ 1.93 (t, 1), 2.41 (s, 3), 2.52 (dt, 2), 4.06 (t, 2), 7.32-7.28 (m, 2), 7.79-7.75 (m, 2).

N-(3-Butynyl)phthalimide

Potassium phythalimide (2.56 g) was added to a mixture of the above tosylate (1.34 g) in 20 mL of DMF. The mixture was heated at 50° C for 6 hours. The mixture was cooled and partitioned between ethyl acetate (2 x 100 mL) and 1M HCl (25 mL) and the organic phases were dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. Purification by flash chromatography (15% ethyl acetate/hexane) gave 1.1 g of the product as a colorless solid.

IR (KBr) 3459, 3253, 1767, 1703, 1469, 1429, 1402, 1371, 1337, 1249, 1210, 1191, 1116, 1088, 996, 868, 795, 726 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.96 (t, 1, J = 2.7), 2.62 (dt, 2, J = 2.7, 7.1), 3.88 (t, 2, J = 7.0), 7.74-7.71 (m, 2), 7.87-7.84 (m, 2); ^{13}C NMR ($CDCl_3$) 18.31, 36.49, 70.23, 80.23, 123.33, 131.94, 134.01, 167.98.

4-Benzyloxycarbonylamino-1-butyne **3d**

Hydrazine hydrate (268 μL) was added to a mixture of the above phthalimide (1.1 g) in 20 mL ethanol, and the mixture was heated at reflux for 1.5 hours. The mixture was cooled to room temperature, and the gummy precipitate was dispersed by adding 1M HCl, and then a colorless solid precipitate formed.

The ethanol was evaporated *in vacuo*, and the solid was filtered out and washed with water. The aqueous phase was lyophilized to give 0.93 g of a colorless solid.

^{1}H NMR (CD_3OD) δ 2.52 (t, 1, J = 2.7), 2.59 (dt, 2, J = 2.7, 6.8), 3.08 (t, 2, J = 6.7); ^{13}C NMR (CD_3OD) δ 17.99, 39.57, 73.11, 79.44.

The solid was dissolved in 40 mL of 50% MeOH/water, and 766 μl of triethylamine was added. A solution of benzyloxycarbonyl succinimide (1.82 g) in 10 mL of MeOH was then added. After 1.5 hours, an additional 1 g of benzyloxycarbonyl succinimide was added and the pH was maintained above 9 by adding triethylamine. After an additional 1.5 hours, the pH was adjusted to 5 by adding 1M HCl. The volatile components were removed *in vacuo*, and the residue was purified by flash chromatography (2.5% MeOH/CH_2Cl_2) to give 0.82 g of the product.

IR (neat) 3416, 3299, 3066, 3034, 2949, 2119, 1703, 1526, 1455, 1367, 1333, 1251, 1216, 1141, 1073, 1021, 1001, 913, 824, 777, 753, 739, 698, 645 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 2.00 (t, 1, J = 2.5), 2.41 (dt, 2, J = 2.3, 6.2), 3.37 (q, 2, J = 6.3), 5.12 (s, 2), 7.32-7.38 (m, 5); ^{13}C NMR ($CDCl_3$) δ 19.77, 39.61, 66.71, 70.00, 128.05, 128.38, 128.44, 136.33, 156.16.

<u>5'-O-tert-Butyldimethylsilyl-2',3'-O-3-N-tris(4-methylbenzoyl)-5-(4-N-carbobenzoyloxyaminobutynyl) uridine **3e**</u>: A solution of Iodo compound **3c** (10 g, 12 mmol), alkyne **3d** (4.8 g, 2 eq), $(Ph_3P)_2PdCl_2$ (200 mg) and CuI (200 mg) in triethylamine (60 mL, deoxygenated) was heated at 50° C for overnight. After completion of the reaction solvent was removed *in vacuo*, the residue was dissolved in chloroform and washed with disodium ethyldiaminetetraacetic acid (5%, 2 x 30 mL), dried, concentrated and the product

was purified by flash chromatography to give compound **3e** as an oil (8 g, 73%, Rf 0.26, ether and methylene chloride 4:96).

^{1}H NMR: 8.20 (s, 1H), 7.90 (d, 2H), 7.80 (t, 4H), 7.38 (m, 5H), 7.22 (t, 4H), 7.12 (d, 2H), 6.60 (d, 1H), 5.72 (d, 1H), 5.69 (t, 1H), 5.40 (t, 1H, NH), 5.16 (bs, 2H), 4.42 (s, 1H), 4.06 (s, 3H), 3.41 (m, 2H), 2.62 (t, 2H), 2.42 (s, 3H), 2.40 (s, 3H), 2.36 (s, 3H), 1.02 (s, 9H), 0.62 (s, 6H).

Preparation of Hydroxy compound **3f**: To a solution of silylcompound **3e** (0.91 g, 1 mmol) in THF (5 mL), a solution of tetrabutylammonium fluoride (1M, 1.2 mL) was added at 0° C under an argon atmosphere and stirred for 2 hours. After completion of the reaction, solvent was removed *in vacuo* and the product was purified by flash chromatography to afford hydroxy compound **3f**.
(0.64 g, 80%, Rf, 0.41, ethyl acetate, hexane 1:1).

^{1}H NMR: 8.46 (s, 1H), 7.92 (m, 6H), 7.32 (m, 6H), 6.40 (d, 1H), 5.82 (m, 2H), 5.32 (bs, 1H), 5.16 (bs, 2H), 4.42 (s, 1H), 3.98 (AB q, 2H), 3.42 (m, 2H), 3.20 (m, 2H), 2.60 (m, 2H), 2.42 (s, 3H), 2.40 (s, 3H), 2.36 (s, 3H).

Dibenzyl 3,4,5-trimethoxyphenylphosphonate **2**

3,4,5-Trimethoxybromobenzene is prepared from 3,4,5-trimethoxybenzoic acid following the procedure of Tetrahedron Lett. 26 (1985):5939-5942. Dibenzyl phosphite is heated in the presence of tetrakis(triphenylphosphine) palladium (0), triethylamine and toluene with 3,4,5-trimethoxybromobenzene to give dibenzyl 3,4,5-trimethoxyphenylphosphonate **2** following the procedure of J. Med. Chem. 32 (1989):1580-1590.

Benzyl 3,4,5-trimethoxyphenylphosphonochloridate **2a**

Phosphorous pentachloride (1.15 mmol) is added to a mixture of diester **2** (1 mmol) in 5 mL of $CHCl_3$. The mixture is heated at 60° C until ^1H NMR of an aliquot shows that no starting material remains (approximately 4 hours). The mixture is cooled to room temperature, and the volatile components are removed *in vacuo* overnight.

Phosphonate ester **3g**

A solution of chloridate **2a** (1 mmol) in 4 mL of CH_2Cl_2 is added to a solution of alcohol **3f** (1 mmol) and DMAP (1.5 mmol) in 4 mL of CH_2Cl_2 at room temperature. When the starting material is consumed as observed by TLC, the solvent is evaporated *in vacuo*. The product is purified by flash chromatography.

5-(4-Aminobutyl)-5'-O-(3,4,5-trimethoxyphenylphosphonyl)uridine **4**

A mixture of nucleoside derivative **3g** (1 mmol) and 5% Pd-C (10 weight %) in 10 mL of methanol is stirred at room temperature under an atmosphere of hydrogen until uptake of hydrogen is complete. The catalyst is removed by filtration through a pad of Celite, washing with methanol. The filtrate is cooled by an ice bath and anhydrous ammonia is bubbled through the solution for 20 minutes. The volatile components are removed *in vacuo*, and the product is purified by reverse phase HPLC.

Example 1c: Preparation of the hapten for prodrug 1a in Example 1a, the linear phosphonate of trimethylbenzoate-5-fluorouridine, Compound 4a.

Refer to Figure 1d for the bold numbered compounds in this Example.

The intermediate phosphochloridate **2d** was prepared starting from bromomesitylene in four steps. Bromomesitylene was treated with n-butyllithium in THF followed by addition of

diethylphosphochloridate which afforded phosphonate compound **2b**. Compound **2b**, on treatment with trimethylsilyl iodide followed by treatment with dilute HCl afforded the corresponding dihydroxy compound **2c**. Compound **2c**, on treatment with PCl_5 in chloroform at 50° C afforded the phosphochloridate **2d**. Compound **3f** was coupled with phosphochloridate **2d** in methylene chloride in the presence of DMAP to afford coupled compound **3h**. Compound **3h** was hydrogenated using Pd-C in ethyl acetate to afford the debenzylated compound which on treatment with aqueous ammonia afforded the hapten **4a**.

In detail, the synthesis is as follows:

Diethyl 2,4,6 trimethylphenyl Phosphonate **2b**: To a solution of 2-bromomesitylene (4 g, 20 mmol) in dry THF (100 mL), a solution of n-butyl lithium (1.6 M, 16 mL) was added dropwise through a syringe under an argon atmosphere at -78° C and stirred for 1 hour. After 1 hour, a solution of phosphochloridate (4.12 g, 1.2 eq) in THF (10 mL) was added and stirred for 1 hour. After completion of the reaction, ammonium chloride solution (10%, 20 mL) was added to the mixture and stirred for 30 minutes. The organic phase was separated, dried, concentrated, and the product was purified by flash chromatography to afford the phosphate **2b** as an oil (1.75 g, 34%, Rf, 0.34, ethyl acetate, hexane, 1:3).

^{1}H NMR: 7. 42 (m, 10H), 6.92 (d, 2H), 4.16 (m, 4H), 2.62 (s, 6H), 2.32 (s, 3H), 1.32 (t, 6H).

Benzyl 2,4,6 trimethylphenyl hydroxy Phosphonate **2c**: A solution of diethylphosphate **2b** (1.5 g, 5.8 mmol) and trimethylsilyl iodide (2.4 g, 12 mmol) in methylene chloride (15 mL) was stirred at 0° C for 1 hour. After completion of the reaction, a solution of sodiumthiosulphate (5%, 5 mL) was added and stirred for 15 minutes. The organic phase was separated, dried and concentrated to give an oily compound. The obtained compound was dissolved in THF (5 mL) and stirred with dil. HCl (5%, 5 mL) for 1 hour. The organic

phase was separated, dried, and concentrated to give the hydroxy compound as an oil (1 g, 85%).

^{1}H NMR: 11.00 (bs, 2H), 7.62 (d, 2H), 3.32 (s, 6H), 2.96 (s, 3H).

A solution of dihydroxy compound (1 g, 5 mmol), benzylalcohol (1.6 g, 3 eq) and trichloroacetonitrile (4.3 g , 6 eq) in pyridine (15 mL) was heated at 75° C overnight under an argon atmosphere. After completion of the reaction, solvent was removed *in vacuo* and the product was purified by flash chromatography to afford the monobenzylated compound **2c** as an oil (0.72 g, 50%, Rf, 0. 36, methanol, methylene chloride, 1:9).

^{1}H NMR: 12.20 (bs, 1H), 7.32 (m, 5H), 6.92 (d, 2H), 5.06 (d, 2H), 2.64 (s, 6H), 2.32 (s, 3H).

Benzyl 2,4,6 trimethylphenyl Phosphonochloridate **2d**: A solution of hydroxy compound **2c** (0.58 g, 2 mmol) PCl_5 (0.56 g, 2 mmol) in chloroform (10 mL) was heated at 50° C for 2 hours. After completion of the reaction, solvent was removed and the compound was dried *in vacuo*.

^{1}H NMR: 7.42 (m, 5H), 6.92 (d, 2H), 5.42 (m, 2H), 2.72 (s, 6H), 2.42 (s, 3H).

Compound **3h**: To a solution of hydroxy compound **3f** (300 mg, 0.38 mmol), DMAP (61 mg, 0.5 mmol) in methylene chloride (3 mL), a solution of phosphochloridate **2d** (151 mg, 1.3 eq) in methylene chloride was added through a syringe under an argon atmosphere. After completion of the reaction solvent was removed *in vacuo* and purification by flash chromatography afforded **3h** (138 mg, 33%, Rf, 0.42, methylene chloride, ethyl acetate, hexane, 3, 3, 4).

^{1}**H NMR**: 7.82 (m, 4H), 7.20 (18H), 6.42 (t, 2H), 6.18 (t, 1H, NH), 5.72 9m, 1H), 5.42 (m, 1H), 5.02 (m, 5H), 4.24 (m, 3H), 3.42 (m , 4H), 2.62-2.40 (singlets of Me, 18H).

5-(4-Aminobutyl)-5'-O-(2,4,6 trimethylphenylphosphonoyl)uridine **4a**: A suspension of compound **3h** (156 mg, 0.14 mmol) in ethyl acetate (2 mL) was stirred in the presence of Pd-C (10%, 15 mg) under hydrogen atmosphere for 2 hours. After completion of the reaction, catalyst was removed by filtration and removal of the solvent afforded the hydrogenated compound (70 mg, 56%).

A solution of this hydrogenated compound (70 mg, 0.08 mmol), ammonium hydroxide (5 mL) in methanol (4 mL) was heated in a sealed tube for overnight. After completion of the reaction solvents were removed *in vacuo* and the product was purified by reverse phase HPLC acetonitrile (1) and water (99) to afford pure compound **4a** as a colorless solid (14 mg, 37%).

^{1}**H NMR**: 7.92 (s, 1H), 6.92 (d, 2H), 6.02 (d, 1H), 4.32 (t, 1H), 4.18 (m, 1H), 4.08 (m, 1H), 3.92 (m, 1H), 3.53 (m , 1H), 3.00 (m , 2H), 2.62 (s, 6H), 2.22 (s, 3H), 2.42 (m, 2H).

Example 2a: Preparation of Prodrug, intramolecular trimethoxybenzoate-5-fluorouridine, Compound 10.

Refer to Figure 2a for the bold numbered compounds in this Example.

The bromobenzoic acid **5**, whose preparation is described in Example 8a, undergoes lithium-halogen exchange and is alkylated with protected iodoethanol **7** (see Figure 2a). The product **8** is dehydrated to form the symmetric anhydride, which is reacted with 5-fluorouridine to

form a stable prodrug precursor, **9**. The protecting group of the precursor can be removed rapidly to give the prodrug **10**.

In detail, the synthesis is as follows:

2-Bromoethyl 4,4'-dimethoxytriphenylmethyl ether 6

DMAP (100 mmol) is added to a solution of 2-bromoethanol (100 mmol) and 4,4'-dimethoxytriphenylmethyl chloride (100 mmol) in DMF (100 mL) at room temperature. After 16 hours, the mixture is poured into water (300 mL) and extracted with ethyl acetate (3 x 100 mL). The organic phases are washed with water (100 mL), dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The mixture is purified by flash chromatography to give the product as a colorless solid.

4,4'-Dimethoxytriphenylmethyl 2-iodoethyl ether 7

A solution of bromide **6** (10 mmol) and NaI (10 mmol) in 100 mL of acetone is heated at reflux with the exclusion of light for 2 hours. The resulting mixture is cooled to room temperature, the solid is removed by filtration, and the solvent is evaporated from the filtrate *in vacuo*. The resulting yellow oil is used without further purification.

2-[2-(4,4'-Dimethoxytriphenylmethoxy)ethyl]-3,4,5-trimethoxybenzoic acid 8

tert-Butyllithium (1.7 M solution in n-pentane, 15 mmol) is added to a solution of bromide **5** (5 mmol) in 50 mL of THF, while maintaining the temperature of the mixture below -95° C. After the addition is completed, the mixture is allowed to warm to -78° C. After 30 minutes, iodide **7** is added in one portion, and the mixture is allowed to warm to 0° C. Water (50 mL) is added, and then the pH of the mixture is carefully adjusted to 3 using 0.1 M HCl. The

mixture is extracted with ethyl acetate (3 x 100 mL). The organic phases are dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The mixture is purified by flash chromatography to give the product as a colorless oil.

5'-O-{2-[2-(4,4'-Dimethoxytriphenylmethoxy)ethyl]-3,4,5-trimethoxybenzoyl}-5-fluorouridine **9**

A solution of DCC (2.5 mmol) in 10 mL of CH_2Cl_2 is added to a solution of acid **8** (5 mmol) in 10 mL of CH_2Cl_2 at room temperature. After 1 hour, the solid is removed from the mixture by filtration, the solid is washed with 5 mL of CH_2Cl_2, and a mixture of 5-fluorouridine (2.5 mmol), and 1-hydroxybenzotriazole (0.25 mmol) in 10 mL of CH_2Cl_2 is added to the combined organic phases. When the reaction is completed as observed by TLC, the mixture is concentrated *in vacuo*. Purification of the mixture by flash chromatography gives the product as a colorless solid.

5'-O-[2-(2-Hydroxyethyl)-3,4,5-trimethoxybenzoyl]-5-fluorouridine **10**

Ether **9** (0.1 mmol) is added in one portion to 80% aqueous acetic acid (10 mL) at room temperature. After 15 minutes, the mixture is poured into saturated $NaHCO_3$ (100 mL) and extracted with ether (3 x 100 mL). The combined organic phases are washed with 100 mL portions of 5% $NaHCO_3$ until no further gas evolution is apparent. The organic phases are then washed with brine (100 mL), dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The mixture is purified by flash chromatography to give the product.

Example 2b: Preparation of the Hapten of Prodrug in Example 2a: The cyclic phosphonate of trimethoxybenzoate-5-fluorouridine, Compound 15.

Refer to Figure 2b for the bold numbered compounds in this Example.

The cyclic phosphonate **13** is synthesized following a typical strategy: bromination, lithiation, hydroxyalkylation, and cyclization of an aryl phosphonate **11**. Saponification of the phosphonate ester, chlorination, and reaction with 2',3'-O-isopropylidene-5-fluorouridine 65 followed by acid hydrolysis with 50% formic acid at 65° C gives the hapten **15**.

In detail, the synthesis is as follows:

Diethyl 3,4,5-trimethoxyphenylphosphonate **11**

Compound **11** is synthesized following the procedure for Compound **2**, using diethylphosphite.

Diethyl 2-bromo-3,4,5-trimethoxyphenylphosphonate **12**

A solution of bromine (10 mmol) in 10 mL of acetic acid is added dropwise to a solution of ester **11** (10 mmol) in 10 mL of acetic acid cooled by an ice water bath. After the red color of the resulting mixture is discharged, the mixture is poured into saturated $NaHCO_3$ (100 mL) and extracted with ethyl acetate (3 x 100 mL). The combined organic phases are washed with 100 mL portions of 5% $NaHCO_3$ until no further gas evolution is apparent. The organic phases are then washed with brine (100 mL), dried over anhydrous $MgSO_4$, and concentrated *in vacuo*. The mixture is purified by flash chromatography to give the product as a pale yellow solid.

Ethyl 2,3-(3,4,5-trimethoxybenzo)butylphostonate **13**

tert-Butyllithium (1.7 M solution in n-pentane, 10 mmol) is added to a solution of bromide **12** (5 mmol) in 50 mL of THF, while maintaining the temperature of the mixture below -95° C. After the addition is completed, the mixture is allowed to warm to -78° C. After 30 minutes, ethylene sulfonate (5 mmol) is added in one portion, and the mixture is allowed to

warm to room temperature. After 1 hour, 1 M HCl (50 mL) is added. After an additional 1 hour, the mixture is extracted with ethyl acetate (3 x 100 mL). The organic phases are dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. The mixture is purified by flash chromatography to give the product as a colorless oil.

2,3-(3,4,5-Trimethoxybenzo)butylphostonic acid **14**

A solution of ester **13** (5 mmol) in 50 mL of methanol at room temperature is maintained at pH 12 with 1 M NaOH until the starting material is consumed, as observed by TLC. The pH is then adjusted to 2 with 1 M HCl and the methanol is evaporated *in vacuo*. The aqueous mixture is extracted with ethyl acetate (3 x 100 mL), and the organic phases are dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. The mixture is purified by flash chromatography to give the product as a colorless oil.

5'-O-[2,3-(3,4,5-Trimethoxybenzo)butylphostonyl]-5-fluorouridine **15**

Thionyl chloride (5 mmol) is added to a solution of acid **14** (5 mmol) in 50 mL of CH_2Cl_2 cooled by an ice water bath. After 1 hour, the volatile components are evaporated *in vacuo*, and the residue is taken up in 10 mL of CH_2Cl_2 and added to a solution of 2',3'-O-isopropylidene-5-fluorouridine **65** (5 mmol) and triethylamine (15 mmol) in 25 mL of CH_2Cl_2 cooled by an ice water bath. After 4 hours, the mixture is poured into 0.1 M HCl (50 mL), the phases are separated, and the aqueous phase is extracted with ethyl acetate (2 x 50 mL). The combined organic phases are dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. The mixture is purified by flash chromatography to give the isopropylidene protected intermediate which on treatment with 50% aqueous formic acid (10 mL) at 65° C for 2 hours and concentration *in vacuo* yields 5'-O-[2,3-(3,4,5-Trimethoxybenzo)-butylphostonyl]-5-fluorouridine **15**.

Example 3: Preparation of Prodrug, galactosyl cytosine b-D-arabinofuranoside, Compound 19.

Refer to Figure 3 for the bold numbered compounds in this Example.

Cytosine β-D-arabinofuranoside was first perbenzoylated and then O-debenzoylated with benzoyl chloride and sodium hydroxide, respectively, to give N^4-benzoyl ara-C **16**. Subsequent coupling with β-galactose pentacetate in the presence of trimethylsilyl trifluoromethanesulfonate in acetonitrile yielded the partially protected compound **17**. Acetylation with acetic anhydride and DMAP in dichloromethane afforded the fully protected compound **18**, which on complete deprotection using ammonia in methanol at 50° C gave the final product, β-gal ara-C **19**.

<u>In detail</u>, the synthesis is as follows:

<u>N^4-Benzoylcytosine-β-D-arabinofuranoside **16**</u>

A suspension of 1.22 g (5.02 mmol) of cytosine-β-D-arabinofuranoside in 50 mL of dry pyridine was cooled to 0° C. 10 mL of benzoyl chloride was added and the mixture was stirred at room temperature for 16 hours. The mixture was poured into 75 mL of 5% aq sodium bicarbonate solution and extracted with CH_2CL_2 (2 x 150 mL). The organic phases were washed with water (50 mL), dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. The mixture was dissolved in 50 mL of pyridine/methanol/water (5:3:2 v/v) and cooled in an ice bath. To this solution was added cold 50 mL of 2M sodium hydroxide in pyridine/methanol/water (5:3:2 v/v). The reaction mixture was stirred at 0° C for 15 minutes and then the pH was adjusted to 7 with the addition of ammonium chloride. The mixture was concentrated *in vacuo* and 20 mL of methanol was added. The mixture was filtered and the solid washed with more methanol (3 x 20 mL). All washings and filtrate were collected,

combined, and concentrated *in vacuo* . Redissolved in 50 mL of methanol/CH_2Cl_2 (2:8 v/v) and the mixture was purified by flash chromatography using methanol/CH_2Cl_2 (1:9-2:8 v/v). A second flash chromatography as described above was needed to remove all impurities to give 1.5 g (86%) of N^4-Benzoylcytosine-β-D-arabinofuranoside **16**.

^1H NMR (D_2O + DMSO-d_6, 2:8 v/v) d 8.2 (1H, d, $J_{5,6}$ 7 Hz, H-6), 7.95 (2H, d, J 7 Hz, *o*-Ph proton x 2), 7.75-7.35 (4H, m, H-5 and Ph proton x 3), 6.1 (1H, d, $J_{1',2'}$ 4 Hz, H-1'), 4.2-3.9 (3H, m, H-2', H-3' and H-4'), and 3.68 (2H, d, $J_{4',5'}$ 4 Hz, H-5').

Coupling reaction; Preparation of Compound **17**

To a solution of galactose penta acetate (1.17 g, 3 mmol) and compound 16 (2 mmol) in dry acetonitrile (5 mL), a solution of trimethylsilyl triflurometnane sulfonate (TMS tf, 354 mg, 1.5 mmol) in dry acetonitrile (2.5 mL) was added through a syringe under argon atmosphere for 2 minutes. Then the reaction mixture was stirred at room temperature for 1 hour and TLC analysis indicated the disappearance of the starting material with the formation of two new compounds (TLC, Ethyl acetate). Then the reaction mixture was quenched with aq. sodium bicarbonate and extracted with ethyl acetate (50 mL). Organic layer was separated, dried, and concentrated to give the colorless solid containing mixture of compounds. The mixture was subjected to flash chromatography to afford Compound **17** (0.8 g, 59% and Rf, 0.36 10% methanol in chloroform).

^1H NMR ($CDCl_3$): 9.60 (bs, 1 H, NH), 8.16 (d, 1 H), 7.86 - 7.42 (m, 6H aromatic and 1 H heterocyclic), 6.20 (d, 1 H), 5.32 (m, 3H, CHO of acetate), 4.62 (d, 1 H, J = 7.6 Hz, anomeric), 4.20 - 3.78 (m, 8H) 3.20 (bs, 1 H), 2.62 (bs, 1 H, 2 x OH, exchanged with D_2O), 2.18 (s, 3 H), 2.04 (s, 6 H), 2.01 (s, 3 H, all CH_3 of acetates).

Compound 17 was peracetylated by using acetic anhydride DMAP in methylene chloride to give the compound **18** (Rf, 0.28, Ethyl acetate twice run, 86%). The product was purified by flash chromatography.

^{1}H NMR ($CDCl_3$): 8.18 (d, 1 H, J = 7.5 Hz), 7.82 - 7.42 (m, 6 H, aromatic, 1 H heterocyclic), 6.42 (d, 1 H, J = 5.1 Hz), 5.62 - 5.08 (m, 5 H, OCH of acetate), 4.60 (d, 1 H, J = 7.8 Hz, anomeric), 4.28 - 3.82 (m, 6 H, OCH), 2.18 (s, 3H), 2.14 (s, 3 H), 2.10 (s, 6 H), 2.06 (s, 3 H), 2 .00 (s, 3 H, all are CH_3 of acetates).

A solution of compound **18** (0.5 g; 0.6 mmol) in methanol (5 ml) and NH_4OH solution (5 ml) was heated at 50° C for 16 hours. TLC analysis indicated the completion of the reaction. Solvents were removed *in vacuo* and the crude product was subject to medium pressure reverse phase C_{18} chromatography using 2% methanol in water as solvents to afford β-gal-Ara-C as a pure colorless solid (0.22 g; 92%)

^{1}H NMR (D_2O): 7.80 (d, 1 H), 6.22 (d, 1 H), 6.02 (d, 1 H), 4.48 (d, 1 H, J = 8.1 Hz, anomeric), 4.40 (t, 1 H), 4.24 (m, 2 H), 3.92 (m, 2 H), 3.80 - 3.60 (m, 6 H).

Example 4: Preparation of Prodrug galactosyl 5-Flurouridine, Compound 24.

Refer to Figure 4 for the bold numbered compounds in this Example.

The synthesis of β-gal 5-fluorouridine **24** follows a similar strategy. 5-Fluorouridine was treated with *t* -butyl dimethylchlorosilane in the prescence of imidazole in DMF at 0° C to give the partially protected compound **20**. Subsequent reaction with acetic anhydride in the prescence of DMAP and triethylamine gave the fully protected nucleoside **21**. Deprotection of the silyl group was achieved using *p* toluene sulphonic acid at 0° C, and the resultant product **22** was coupled with β-galactose pentacetate in the presence of trimethylsilyl

trifluoromethanesulfonate in acetonitrile to give the fully protected compound **23**. Complete deprotection with ammonia in methanol at 50° C afforded the final product, β-gal 5-fluorouridine **24**.

<u>In detail</u>, the synthesis is as follows:

Preparation of Compound **21**: To a cooled solution of 5-Flurouridine (1.31 g, 5 mmol) in DMF sequentially added imidazole (0.816 g, 12 mmol) and *t*-butyldimethylchlorosilane (0.90 g, 6 mmol) and contents were stirred at 0° C for 2 hours. After completion of the reaction (TLC, 10% Methanol in chloroform) contents were transferred into a separating funnel containing ethyl acetate (100 mL), washed with water (3 times, 25 mL each) and organic layer was separated, dried (MgSO4) and concentrated to give monosilylated product **20** as an oily compound (Rf, 0.44, 10% methanol in chloroform).

The above obtained product **20** (1.80 g, crude, 5 mmol) was dissolved in methylene chloride (20 mL) and added sequentially DMAP (1.34 g, 11 mmol) and acetic anhydride (1.22 g, 12 mmol) and reaction mixture was stirred at room temperature for 1.5 hours. TLC analysis (1:1 Ethyl acetate: Hexane) indicated the completion of reaction. Then the reaction mixture was transferred into a separating funnel and washed with water, dried, concentrated and the product was purified by flash chromatography to afford compound **21** in pure form (Rf, 0.48, 1:1 EtOAc and Hexane, 1.90 g, 83%).

^{1}H NMR ($CDCl_3$); 8.02 (d, 1 H), 6.26 (d, 1 H), 5.34 (m, 2 H, CH0 of acetate), 4.22 (m, 1 H), 3.86 (AB q, 2 H), 2.08, 2 .04, (2 x s, 3 H, each, CH_3 of acetate), 0.92 (s, 9 H, t-bu si), 0.12 (s, 6 H, CH_3 of silyl).

^{13}C HNMR ($CDCl_3$): 169.99, 169.72, 157.06, 156.71, 149.61, 142.51, 139.35, 85.46, 84.12, 73.25, 71.94, 63.28, 25.74, 20.70, 20.68, 20.37, 18.37, -5.70.

Preparation of Compound **22**: To a cooled solution (0° C) of compound **21** (1.69 g, 3.5 mmol) in methanol (6 mL) and methylene chloride (12 mL), catalytic amount of PTSA (100 mg) was added and reaction mixture was stirred at 0° C for 30 minutes. After completion of reaction (TLC) it was quenched with triethylamine (0.5 mL) and removed the solvents to give crude compound **22** as an oil. It was then chromatographed to give compound **22** in pure form (Rf, 0.22, 1:1 EtOAc and Hexane, 920 mg 76%).

^{1}H NMR ($CDCl_3$): 8.09 (d, 1 H J = 6.3 Hz), 6.14 (d, 1 H), 5.43 (m 2 H, CHO of acetate), 4.21 (m, 1 H), 3.86 (AB q, 2 H), 2.08, 2.04 (2 x s, 3 H each, CH_3 of acetate).

^{13}C HNMR ($CDCl_3$): 170.34, 170.08, 157.56, 149.55, 139.17, 86.61, 83.72, 73.21, 71.48, 61.65, 20.65, 20.38.

Preparation of coupling compound **23**: Coupling reaction between galactose penta acetate and compound **22** was accomplished by the method as mentioned above to give coupling product **23** (Rf, 0.20, 1:1 EtOAC: Hexane, 59%).

^{1}H NMR ($CDCl_3$): 9.60 (bs, 1 H, NH), 8.18 (d, 1 H, J = 6.6 Hz), 6.34 (m, 1 H), 5.46 - 5.08 (m, 5 H, OCH of acetates), 4.61 (d, 1 H, J = 8.1 Hz, anomeric), 4.30 - 3.72 (m, 6 H), 2.16, 2.13, 2.11, 2.09, 2.05, 2.01 (6 x s, each 3 H, CH_3 of acetate).

^{13}C HNMR: 170.37, 170.10, 169.34, 157.00, 156.64, 149.43, 142.52, 139.37, 100.44, 85.83, 82.35, 73.35, 71.63, 70.35, 70.34, 68.57, 68.11, 66.74, 61.13, 20.34, 20.07, 20.29.

Preparation of Prodrug β-D-Gal Fluorouridine **24**: Compound **23** was converted to prodrug, compound **24** via the same method (ammonia) as described above (92%).

^1H NMR (D_2O): 8.12 (d, 1 H), 5.88 (d, 1 H), 4.44 (d, 1 H, J =7 Hz anomeric), 4.36 - 3.62 (m, 11 H).

Example 5a: Preparation of the precursor to the Hapten of the Prodrugs in Examples 3 and 4, Compound 25.

Refer to Figure 5a for the bold numbered compounds in this Example.

The aminonucleoside **25** is prepared from 5-fluorouridine according to Scheme in Figure 5a. Compound **22** (Figure 4) is activated with triphenylphosphine and carbontetrabromide, and is then subsequently treated with sodium azide to form an azide intermediate. This intermediate is then hydrogenated with 10% Pd-C to an amine which is then deprotected with sodium methoxide in methanol to give the aminonucleoside **25**. This aminonucleoside is used in subsequent coupling reactions to give the amidine compound **30b** (R=5-fluorouridine).

In detail, the synthesis is as follows:

Preparation Of 5'-Amino-5-fluororidine **25**

To a solution of 5'-hydroxy 2', 3' diacetoxy-5-fluorouridine **22** (1 eq) in methylene chloride (0.2 M) are added sequentially triphenyl phosphine (1.1 eq), and carbon tetrabromide (1.2 eq) and the mixture is stirred at 0° C. After completion of the reaction the product is ready for the next step.

The crude bromide compound (1 eq) is then dissolved in DMF (0.2 M) and heated with sodium azide (3 eq) at 60°. After the completion of the reaction, the reaction mixture is

transferred to a separating funnel containing ethyl acetate. The solution is washed with water and the organic layer is separated, dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. The crude product is purified by flash chromatography to give the azide derivative which is the precursor of compound **25**.

The azide derivative is dissolved is ethyl acetate and 10% palladium on activated carbon (0.05 eq) is added. To this stirred suspension an atmosphere of hydrogen is placed using a balloon filled with hydrogen gas. After completion of the reaction, the catalyst is removed by filtering through a bed of celite and the filtrate is collected and concentrated to give the corresponding amino precursor of compound **25**.

The amino precursor is dissolved in methanol and sodium methoxide (0.05 eq) is added. The solution is stirred at room temperature and at the completion of the reaction glacial acetic acid (0.05 eq) is added and the mixture is concentrated *in vacuo*. Compound 25 is purified by medium pressure reverse phase C_{18} chromatography using methanol in water as solvent.

Example 5b: Preparation of the Hapten of the Prodrugs in Examples 3 and 4, Compounds 30a and 30b.

Refer to Figure 5b and 5c for the bold numbered compounds in this Example.

The preparation of the amidine Compound **30a** and/or **30b** (R= ara C or 5-fluorouridine) can be accomplished by two different synthetic routes. One synthetic route starts with the commercially available diacetone D glucose (Figure 5b, described here in Example 5b) whilst the other starts from glucospyranose (Figure 5c, described here in Example 5c).

Starting from diacetone D glucose, the first step involves silylation of the hydroxy group with *t*-butyl dimethylchlorosilane in the prescence of imidazole in DMF. Subsequent treatment

with aqueous acetic acid affords the 5,6 diol which is then silylated at the primary hydroxy position with *t*-butyl dimethylchlorosilane and the remaining secondary hydroxy group is converted to a mesylate on treatment with MsCl in the prescence of triethylamine. The resultant mesylate compound **26** is then converted to the azide compound **27** by first reacting it with sodium iodide in acetone and then treating the iodide derivative with sodium azide in DMF. Hydrolysis of the acetonide group is accomplished by treating compound **27** with aqueous acetic acid at 60° C. The resultant diol is then oxidised at the anomeric position with bromine in aqueous dioxane to give a lactone derivative which is subsequently silylated with *t*-butyldimethylchlorosilane to give the lactone compound **28**. The azide group of compound **28** is converted to an amino group when subjected to hydrogenation using 10% Pd on carbon, and as a result, rearranges to a glucolactam **29a** derivative. Inversion of the secondary hydroxy group using the Mitsunobu reaction procedure gives the galactolactam **29b** derivative. Activation with Meerweins reagent and subsequent coupling with the amino nucleoside **25** (Example 5a) followed by desilylation with fluoride gives the final amidine compound **30b** (R= 5-fluorouridine).

In detail, the synthesis is as follows:

Preparation of Compound 26

To a mixture of diacetone D-glucose (5.2 g, 20 mmol) in dry DMF (50 ml), sequentially added imidazole (3.26 g, 48 mmol) *t*-butyl dimethylchlorosilane (3.60 g, 24 mmol) and contents are stirred at room temperature for 4 hours. After completion of the reaction, the reaction mixture is transferred to a separatory funnel containing ethyl acetate (250 mL) and washed with water, dried, concentrated and product is purified by flash chromatography.

The silylated compound (6.73 g, 17.9 mmol) obtained is dissolved in THF (50 mL) and stirred with aq acetic acid (6 mL) for 6 hours. After completion of the reaction (TLC) solvent was removed and the product is purified by flash chromatography.

The above obtained diol (5.38 g, 16.1 mmol) is dissolved in dry DMF (60 mL) and sequentially added imidazole (2.62 g, 2.4 eq) and tbutyl dimethylchlorosilane (1.2 Eq) and stirred at 0° C for 2 hours. After completion of the reaction, it is dissolved in ethyl acetate (200 mL) and washed with water, dried, concentrated and the product is purified by chromatography.

The monosilylated hydroxy compound (5.6 g, 12.5 mmol) is dissolved in methylene chloride (40 mL) and cooled to 0° C and sequentially added triethylamine (2.7 mL) and MsCl (1.85 g, 1.3 eq) and contents are stirred at 0° C for 3 hours. After completion of the reaction, it is transferred into a separatory funnel and washed with water, dried, and concentrated to give the corresponding mesylate compound **26**.

Preparation of Azide **27**: A mixture of mesylate **26** (5.26 g, 10 mmol), sodium iodide (1.93 g, 13 mmol) in acetone (50 mL) is heated at reflux for 4 hours. After completion of the reaction, solvent is removed and the resulting material is dissolved in ethyl acetate (100 mL) and washed with water, dried, and the product is purified by chromatography.

A mixture of iodide (4.54 g, 8 mmol), sodium azide (1.30 g, 20 mmol) in dry DMF was heated at 60° C for 6 hours. After completion of the reaction, it is diluted with ethyl acetate (200 mL) and washed with water, dried and concentrated. The product is purified by chromatography to obtain azide **27** as a pure compound.

Preparation of Lactone **28**: The obtained azide **27** (2.89 g, 6 mmol) is dissolved in THF (30 mL) and aq acetic acid (10 mL) and contents are heated at 60° C for 6 hours. After

completion of the reaction solvent is removed and the resulting material is dissolved in ethyl acetate dried and concentrated to give the diol.

A bromine (1 eq) solution in dioxane was added to the above obtained diol (1.77 g, 4 mmol) in aq dioxane (10%, 20 mL) and the resulting mixture is stirred at room temperature for 2 hours. After completion of reaction, it is diluted with ethyl acetate (50 mL) and washed with aq sodium thiosulfate, dried and concentrated to give hydroxy lactone.

The hydroxyl group in the above lactone is protected as *t*-butyldimethyl silyl ether as described previously to obtain the lactone **28**.

Preparation of Lactam **29a**: A suspension of azide **28** (1.10 g, 2 mmol) in methanol (10 mL) and Pd-C (10%, 110 mg) is hydrogenated using hydrogen balloon for 4 hours. After completion of reaction, catalyst was filtered through celite and solvent is removed to give lactam **29a**.

Preparation of Lactam having Galacto Configuration **29b**: The above obtained lactam **29a** was converted to the galacto lactam **29b** as mentioned below. To a solution of lactam **29a** (0.86 g, 1.6 mmol) and acetic acid (2 mL) in methylene chloride (8 mL) are added sequentially triphenyl phosphine (0.419 g, 1.6 mmol) and diethyl azodicarboxylate (0.295 g 1.7 mmol) at 0° C and the reaction mixture is stirred for 2 hours. After completion of reaction the solvent is removed and the product is isolated by chromatography. The obtained acetate is hydrolysed by sodium methoxide to obtain the galacto lactam **29b**.

A mixture of galactolactam **29b** (1 eq), Meerweins reagent (triethyloxonium tetrafluoroborate, 1 M solution in dichloromethane, 1.2 eq) in dichloromethane is stirred at room temperature for 1 hour. The aminonucleoside **25** (1 eq) is then added and when the reaction is complete, the product is purified by flash chromatography.

Example 5c: The alternative preparation of the Hapten of the Prodrugs in Examples 3 and 4, Compounds 30a and 30b.

Refer to Figure 5c for the bold numbered compounds in this Example.

Preparation of the galactose-β-5-fluorouridine amidine using 5-fluorouridine starts with commercially available glucopyranose **31**. Treatment with 2,2 dimethoxypropane in acetone in the presence of catalytic amount of *p* toluenesufonic acid gives the protected compound **32**. Further protection of the remaining hydroxy groups with *t*-butyldimethylchlorosilane affords the fully protected compound **33**. Heating to reflux with benzyl alcohol opens the lactone and the resultant hydroxy compound **34** is mesylated with MsCl. Subsequent conversion to the azide compound **35** is achieved in two steps by first reacting the mesylate group with sodium iodide in acetone and then displacing the iodide group with an azide group using sodium azide in DMF. Hydrogenation using 10% Pd on carbon converts the azide group to an amino which then cyclises to a lactam . Deprotection of the acetonide to a diol on treatment with trifluoroacetic acid and subsequent protection of the primary alcohol with *t*-butyl dimethylchlorosilane yields the glucolactam compound **29a**. The secondary hydroxy group is inverted using the Mitsunobu reaction procedure and the subsequent activation and coupling of the amide is accomplished using Meerweins reagent and the aminonucleside **25** (Example 5a) respectively. Final deprotection using fluoride yields the amidine compound **30b** (R_2= 5-fluorouridine).

In detail, the synthesis is as follows:

Preparation of Lactone **32**: A mixture of hydroxy compound **31** (8.90 g, 50 mmol), 2, 2 dimethoxy propane (4 eq) and PTSA (0.5 g) in methylene chloride (400 mL) and acetone (100 mL) is stirred for 4 hours. After completion of the reaction, it is quenched with

triethylamine (3 mL) and the solvent is removed and resulting crude compound is purified by chromatography to obtain compound **32**.

Preparation of Silyl Compound **33**: A mixture of compound **32** (8.72 g, 40 mmol), imidazole (4.4 eq) and *t*-butyl dimethylchlorosilane (2.2 eq) in dry DMF is stirred for 6 hours. After completion of the reaction, it is diluted with ethyl acetate (500 mL), and washed with water (100 mL x 2), dried concentrated and the product is isolated by chromatography to obtain compound **33**.

Preparation of Compound **34**: A solution of compound **33** (13.38 g, 30 mmol) in a mixture of Benzyl alcohol (100 mL) and chloroform (300 mL) is heated at 60° C until the reaction is completed. After completion of the reaction, solvents are removed and the product is isolated by chromatography to give compound **34**. When methanol is used it gives the corresponding hydroxy methyl ester.

Preparation of Azido Ester **35**: Conversion of hydroxy compound **34** to azido compound **35** is achieved by the same sequence of reactions as used for the preparation of hydroxy compound **26** to compound **27**, to obtain azido compound **35**.

Preparation of Lactam **29a** and Lactam **29b**: Hydrogenation of Compound **35** (under previously described conditions) and deprotection using trifluoroacetic acid (*vide supra*) and primary alcoholic protection gives **29a**. Lactam **29a** is inverted to galacto lactam **29b** using Mitsunobu reaction condition (*vide supra).*

Conversion of compound **29a** to amidine compound **30b** can be accomplished using the same procedure described previously (*vide supra*).

Example 6: Preparation of the Prodrug, aliphatic diethyl acetal protected aldophosphamide, Compound 38.

Refer to Figure 6 for the bold numbered compounds in this Example.

When bis(2-chloroethyl)amine hydrochloride was heated with an excess of phosphorus oxychloride, the dichlorophosphamide **36** was obtained after distillation as a crystalline solid in good yield. Reaction of the dichlorophosphamide **36** with one molar equivalent of 3-hydroxypropionaldehyde diethyl acetal gave monochlorophosphamide **37** which on treatment with ammonia afforded 3,3-diethoxypropionyl N,N-bis(2-chloroethyl)phosphoric diamide **38**.

In detail, the synthesis is as follows:

N,N-Bis(2-chloroethyl)phosphoramidic dichloride **36**

A mixture of bis(2-chloroethyl)amine hydrochloride (5 g) and $POCl_3$ (13 mL) was heated at reflux for 12 hours, during which the mixture became a homogeneous solution. The excess $POCl_3$ was removed by distillation (bp 105° C), and then 4.93 g of the product was distilled (bp 110-114° C, 0.1 mm Hg). Recrystalliztion from acetone/hexane gave 4.5 g of the product as a colorless solid: mp 54.5-56° C (lit. 54-56° C, Friedman, O. M., et. al., J. Am. Chem. Soc. 76 (1954):655-658); ^1H NMR ($CDCl_3$) δ 3.62-3.68 (m, 2), 3.71-3.77 (m, 6).

3,3-Diethoxypropionyl N,N-bis(2-chloroethyl)phosphoramidic chloride **37**

A solution of dichloridate 36 (1.75 g) in 5 mL of CH_2Cl_2 was added dropwise to a mixture of 3,3-diethoxy-1-propanol (1.0 g) and DMAP (0.91g) in 10 mL of CH_2Cl_2. After 19 hours, a precipitate was formed. The precipitate was removed by filtration, and the volatile

components were removed *in vacuo*. The residue was passed through a short column of silica gel, eluting with 25% and then 30% ethyl acetate/hexane, to give 0.95 g of the product: Rf 0.38 (25% ethyl acetate/hexane); ^1H NMR ($CDCl_3$) δ 1.23 (t, 6), 2.06 (q, 2), 3.40-3.60 (m, 6), 3.62-3.75 (m, 6), 4.21-4.38 (m, 2), 4.62 (t, 1).

3,3-Diethoxypropionyl N,N-bis(2-chloroethyl)phosphoric diamide **38**

Anhydrous ammonia was bubbled through a solution of chloridate **37** (0.95 g) in 10 mL of CH_2Cl_2 for 20 minutes, resulting in the formation of a precipitate. The solid was removed by filtration, and the volatile components were removed *in vacuo* to give 0.71 g of an oil. A portion (100 mg) of the crude product was passed through a short column of silica gel, eluting with 3% Et_3N in 50% ethyl acetate/hexane, to give 46 mg of the product as a colorless oil: Rf 0.16 (3% Et_3N in 30% ethyl acetate/hexane); IR ($CDCl_3$) 3600-3100, 2976, 2932, 2849, 1573, 1446, 1376, 1348, 1225, 1132, 1056, 985, 752, 657 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.20 (t, 6), 1.95 (q, 2), 3.34-3.75 (m, 12), 4.00-4.15 (m, 2), 4.63 (t, 1).

Stability of acetal **38**

A sample of acetal **38** was dissolved in 0.9 weight % NaCl in D_2O at room temperature. No change in the ^1H NMR spectrum was observable after 2 days.

Example 7: Preparation of the guanyl Hapten of the Prodrug, aliphatic diethyl acetal protected aldophosphamide, Compound 43.

Refer to Figure 7 for the bold numbered compounds in this Example.

N-*t*-Boc-aminoethylphosphonic acid **39** is prepared by the reaction of 2-aminophosphonic acid with di-*tert*-butyl dicarbonate. Subsequent reaction with bis(2-chloroethyl)amine

hydrochloride in the presence of triethylamine, 4-dimethylaminopyridine and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride affords the phosphoramidic acid **40**. Conversion to the phosphoric diamide **41** is achieved by first activating with 1-(2-mesitylenesulfonyl)-3-nitro-1,2,4-triazole and then reacting with ammonia. Deprotection with trifluoroacetic acid gives **42**, which reacts with N,N-diethyl-O-methylisourea tetrafluoroborate to yield the final guanidinium product **43**.

In detail, the synthesis is as follows:

N-*t*-Boc-2-aminoethylphosphonic acid **39**

1.25 g of 2-aminoethylphosphonic acid and 4.2 mL of triethylamine are dissolved in 10 mL of water and a solution of 2.62 g of di-*tert*-butyl dicarbonate in 10 mL of dry acetonitrile is added. The pH is kept at 9 by addition of triethylamine. After the addition is complete, the mixture is stirred for 2 hours and then concentrated *in vacuo*. The residue is redissolved in 0.01 M $NaHCO_3$ (100 mL) and washed with ethyl acetate (2x50 mL). The aqueous phase is adjusted to pH 1 by addition of 0.1 M HCl and extracted with ethyl acetate (2x100 mL). The organic phases are dried over anhydrous $MgSO_4$ and concentrated *in vacuo* to give N-*t*-Boc-2-aminoethylphosphonic acid **39**.

N,N-Bis(2-chloroethyl)-P-[N'-(*t*-Boc)-2-aminoethyl]phosphonamidic acid **40**

A mixture of 2.25 g of N-*t*-Boc-2-aminoethylphosphonic acid **39**, 2.14 g of bis(2-chloroethyl)amine hydrochloride, 4.2 mL of triethylamine and 0.146 g of 4-dimethylaminopyridine are dissolved in 20 mL of DMF/CH_2Cl_2 (1:1 v/v). 2.3 g of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride is added and the mixture is stirred at room temperature for 16 hours. The mixture is poured into 1 M NaOAc, pH 5 (75 mL) and washed with ether (2x75 mL). The aqueous phase to pH 1 with 1 M HCl and

immediately extracted with ethyl acetate (2x100 mL). The organic phases are washed with water (20 mL), dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. The mixture is purified by flash chromatography to give N,N-bis(2-chloroethyl)-P-[N'-(*t*-Boc)-2-aminoethyl]phosphonamidic acid **40**.

N,N-Bis(2-chloroethyl)-P-[N'-(*t*-Boc)-2-aminoethyl]phosphonic diamide **41**

1.75 g of N,N-bis(2-chloroethyl)-P-[N'-(*t*-Boc)-2-aminoethyl]phosphonamidic acid **40** is dissolved in dry pyridine (50 mL) and concentrated *in vacuo*. This process is repeated once more with more pyridine (50 mL). The residue is dissolved in dry pyridine (25 mL) and 2.96 g of 1-(2-mesitylenesulfonyl)-3-nitro-1,2,4-triazole is added. Ammonia gas is bubbled through for 60 minutes. The reaction mixture is concentrated *in vacuo*, redissolved in ethyl acetate (100 mL) and washed with saturated $NaHCO_3$ (2x100 mL) and saturated NaCl (75 mL). The organic phase is dried over anhydrous $MgSO_4$, concentrated *in vacuo* and purified by flash chromatography to yield N,N-bis(2-chloroethyl)-P-[N'-(t-Boc)-2-aminoethyl]phosphonic diamide **41**.

N,N-Bis(2-chloroethyl)-P-[2-(2,3-diethylguanidyl)ethyl]phosphonic diamide **43**

0.873 g of N,N-bis(2-chloroethyl)-P-[N'-(t-Boc)-2-aminoethyl]phosphonic diamide **41** is dissolved in 10 mL of dichloromethane and 10 mL of trifluoroacetic acid is added. After 60 minutes the reaction mixture is concentrated *in vacuo* to give **42**. The residue is redissolved in a mixture of 1 mL of triethylamine and 20 mL of water. The pH is adjusted to 8.5 with more triethylamine and 0.872 g of N,N'-diethyl-O-methylisourea tetrafluoroborate is added while keeping the pH at 8.5 with triethylamine. After 16 hours the reaction mixture is adjusted to pH 7 with acetic acid and concentrated *in vacuo*. The residue is redissolved in 5 mL of water and purified using reverse phase ODS chromatography to give N,N-bis(2-chloroethyl)-P-[2-(2,3-diethylguanidyl)ethyl]phosphonic diamide **43**.

Example 8a: Preparation of the anhydride intermediate, Compound 45, for the synthesis of intramolecular enol trimethoxybenzoate phosphamide prodrug.

Refer to Figure 8a for the bold numbered compounds in this Example.

Commercially available trimethoxybenzoic acid is brominated and the product **5** undergoes low temperature lithium-halogen exchange to produce the aryllithium intermediate. The reactive intermediate is alkylated by a protected iodoethanol **7**, and the product **44** is dehydrated to form the symmetric anhydride **45**.

In detail, the synthesis is as follows:

2-Bromo-3,4,5-trimethoxybenzoic acid **5**

A solution of bromine (100 mmol) in 100 mL of acetic acid is added dropwise to a solution of 3,4,5-trimethoxybenzoic acid (100 mmol) in 100 mL of acetic acid cooled by an ice water bath. After the red color of the resulting mixture is discharged, the mixture is poured onto 500 g of crushed ice. The resulting solid is collected by filtration, dried over P_2O_5 *in vacuo*, and recrystallized from Et_2O to give the product as a pale yellow solid.

2-[2-(4,4'-Dimethoxytriphenylmethoxy)ethyl]-3,4,5-trimethoxybenzoic acid (**44**)

tert-Butyllithium (1.7 M solution in n-pentane, 15 mmol) is added to a solution of bromide **5** (5 mmol) in 50 mL of THF, while maintaining the temperature of the mixture below -95° C. After the addition is completed, the mixture is allowed to warm to -78° C. After 30 minutes, iodide **7** (synthesis described in Example 2a) is added in one portion, and the mixture is

allowed to warm to 0° C. Water (50 mL) is added, and then the pH of the mixture is carefully adjusted to 3 using 0.1 M HCl. The mixture is extracted with ethyl acetate (3 x 100 mL). The organic phases are dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The mixture is purified by flash chromatography to give the product as a colorless oil.

<u>2-[2-(4,4'-Dimethoxytriphenylmethoxy)ethyl]-3,4,5-trimethoxybenzoic anhydride (45)</u>

A solution of DCC (5.5 mmol) in 10 mL of CH_2Cl_2 is added to a solution of acid **44** (10 mmol) in 25 mL of CH_2Cl_2 at room temperature. After 1 hour, the resulting solid is removed by filtration and washed with 25 mL of CH_2Cl_2, and the solvent is evaporated from the filtrate *in vacuo*. The product is used without further purification. This anhydride is used in Example 8b to synthesize the aldophosphamide prodrug compound **50** Figure 8b.

Example 8b: Preparation of the Prodrug, intramolecular enol trimethoxybenzoate phosphamide, Compound 50.

Refer to Figure 8b for the bold numbered compounds in this Example.

The previously prepared symmetric anhydride **45** (Example 8a) is reacted with a β-siloxy propanal enolate to form the enol benzoate **48**. The silyl protecting group is removed, and the alcohol thus revealed is reacted with a phosphoramide dichloridate followed by ammonia to form a relatively stable prodrug precursor **49**. This precursor **49** can be rapidly transformed into the more reactive prodrug **50**, as needed.

<u>In detail</u>, the synthesis is as follows:

<u>3-(*tert*-Butyldimethylsiloxy)-1-propanol (46)</u>

A mixture of 1,3-propanediol (10 mmol), *tert*-butyldimethylchlorosilane (11 mmol), and imidazole (22 mmol) dissolved in 5 mL of DMF was stirred at room temperature for 16 hours. The mixture was poured into 0.1 M HCl (100 mL) and extracted with ether (3 x 100 mL). The organic phases were washed with brine (100 mL), dried over anhydrous $MgSO_4$, and concentrated *in vacuo*. The mixture was purified by flash chromatography to give the product as a colorless oil.

3-(*tert*-Butyldimethylsiloxy)propanal (**47**)

DMSO (24 mmol) is added to oxalyl chloride (11 mmol) in 40 mL of CH_2Cl_2 cooled to -78° C. After 15 minutes, alcohol **46** (10 mmol) is added. After an additional 15 minutes, triethylamine (50 mmol) is added. The mixture is allowed to warm to 0° C and then poured into 0.1 M HCl (100 mL). The phases are separated, and the aqueous phase is extracted with ethyl acetate (2 x 100 mL). The organic phases are washed with water (100 mL), dried over anhydrous $MgSO_4$, and concentrated *in vacuo*. The mixture is purified by flash chromatography to give the product as a colorless oil.

3-*tert*-Butyldimethylsiloxyprop-1-enyl 2-[2-(4,4'-dimethoxytriphenylmethoxy)ethyl]-3,4,5-trimethoxybenzoate (**48**)

NaH (60% dispersion in mineral oil, 11 mmol) is washed with hexane (2 x 10 mL). Ether (20 mL) is added, followed by the dropwise addition of a solution of aldehyde **47** (10 mmol) in 20 mL of ether. Fifteen minutes after the addition is completed, a solution of anhydride **45** (20 mmol) in 20 mL of ether is added in one portion. After an additional 0.5 hour, the reaction mixture is poured into saturated NH_4Cl (20 mL) and the phases are separated. The aqueous phase is extracted with ether (3 x 40 mL). The combined organic phases are extracted with brine (40 mL), dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The mixture is purified by flash chromatography to give the product as a colorless oil.

3-{2-[2-(4,4'-Dimethoxytriphenylmethoxy)ethyl]-3,4,5-trimethoxybenzoyloxy}prop-2-enyl N,N-bis(2-chloroethyl)phosphoric diamide (49)

Tetrabutylammonium fluoride (1.0 M solution in THF, 2 mmol) is added to a solution of silyl ether **48** (2 mmol) in 50 mL of THF cooled to -23° C. After 5 minutes, triethylamine (2 mmol) is added, followed by **36** (2 mmol, synthesis described in Example 6). After an additional 3 hours, NH_3 is added. After a further 2 hours, the reaction mixture is poured into ice-cold brine and extracted with ether (4 x 100 mL). The combined organic phases are dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. Purification by flash chromatography gives the product as a colorless oil.

3-[2-(2-Hydroxyethyl)-3,4,5-trimethoxybenzoyloxy]prop-2-enyl N,N-bis(2-chloroethyl)phosphoric diamide (50).

Trityl ether **49** (0.1 mmol) is added in one portion to 80% aqueous acetic acid (10 mL) at room temperature. After 15 minutes, the mixture is poured into saturated $NaHCO_3$ (100 mL) and extracted with ether (3 x 100 mL). The combined organic phases are washed with 100 mL portions of 5% $NaHCO_3$ until no further gas evolution is apparent. The organic phases are then washed with brine (100 mL), dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The mixture is purified by flash chromatography to give the product as colorless solid.

Example 8c: Preparation of the intramolecular enol trimethoxybenzoate phosphamide hapten, Compound 57.

Refer to Figure 8c for the bold numbered compounds in this Example.

The protected aryl phosphite **51** is synthesized following literature precedent. The aromatic ring is brominated, the bromide **52** undergoes lithium-halogen exchange, and the aryllithium so produced is hydroxyethylated to give **53**. After workup and deprotection, the cyclic phosphite **54** is obtained. The phosphite **54** undergoes the Perkow reaction with the α-bromo aldehyde **55** to produce the enol phosphonate **56**. Deprotection and reaction with phosphorus oxychloride, followed by N-trifluoroacetylpiperazine and then ammonia gives the hapten **57**, which can be linked to a carrier protein through the piperazine ring.

In detail, the synthesis is as follows:

Ethyl P-(3,4,5-trimethoxyphenyl)-P-(diethoxymethyl)phosphinate (51)

3,4,5-Trimethoxybromobenzene is prepared following the procedure of Tetrahedron Lett. 26 (1985): 5939-5942 incorporated herein by reference. Ethyl (diethoxymethyl)phosphonite is prepared following the procedure of Tetrahedron 45 (1989):3787-3808, incorporated herein by reference, and reacted with 3,4,5-trimethoxybromobenzene following the procedure of J. Med. Chem. 32 (1989):1580-1590, incorporated herein by reference.

Ethyl P-(2-bromo-3,4,5-trimethoxyphenyl)-P-(diethoxymethyl)phosphinate (52)

A solution of bromine (10 mmol) in 10 mL of acetic acid is added dropwise to a solution of ester **51** (10 mmol) in 10 mL of acetic acid cooled by an ice water bath. After the red color of the resulting mixture is discharged, the mixture is poured into saturated $NaHCO_3$ (100 mL) and extracted with ethyl acetate (3 x 100 mL). The combined organic phases are washed with 100 mL portions of 5% $NaHCO_3$ until no further gas evolution is apparent. The organic phases are then washed with brine (100 mL), dried over anhydrous $MgSO_4$, and concentrated *in vacuo*. The mixture is purified by flash chromatography to give the product as a pale yellow solid.

P-Diethoxymethyl-2,3-(3,4,5-trimethoxybenzo)butylphostinate (53)

tert-Butyllithium (1.7 M solution in n-pentane, 10 mmol) is added to a solution of bromide **52** (5 mmol) in 50 mL of THF, while maintaining the temperature of the mixture below -95° C. After the addition is completed, the mixture is allowed to warm to -78° C. After 30 minutes, ethylene sulfonate (5 mmol) is added in one portion, and the mixture is allowed to warm to room temperature. After 1 hour, 1 M HCl (50 mL) is added. After an additional 1 hour, the mixture is extracted with ethyl acetate (3 x 100 mL). The organic phases are dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. The mixture is purified by flash chromatography to give the product as a colorless oil.

2,3-(3,4,5-Trimethoxybenzo)butylphostinic acid (54)

A mixture of compound **53** (5 mmol) in 20 mL of 36% aqueous HCl is heated at 100° C for 2 hours. After cooling to room temperature, the reaction mixture is diluted with 200 mL of water and extracted with ethyl acetate (4 x 100 mL), and the organic phases are dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. The mixture is purified by flash chromatography to give the product as a colorless oil.

2-Bromo-3-*tert*-butyldimethylsiloxypropanal (55)

A mixture of $CuBr_2$ (10 mmol) and aldehyde **47** (10 mmol) in ethyl acetate (50 mL) and chloroform (50 mL) is heated at reflux for 6 hours with the exclusion of light. The mixture is cooled to room temperature, the solid is removed by filtration and washed with ethyl acetate (50 mL), and the combined organic phases are dried over $MgSO_4$ and concentrated *in vacuo*. Purification of the mixture by flash chromatography gives the product as a pale yellow oil.

3-*tert*-Butyldimethylsiloxy-1-propenyl 2,3-(3,4,5-trimethoxybenzo)butylphostonate (56)

Acid **54** (1 mmol) is dissolved in hexamethyldisilazane (1 mL) and heated at reflux for 3 hours. The mixture is cooled to room temperature and the volatile components are evaporated *in vacuo*. Aldehyde **55** (1 mmol) is added to the resulting oil and the mixture is heated at 100° C under a slow stream of nitrogen for 4 hours. After cooling, the mixture is purified by flash chromatography.

3-[P-amino-P-(N-piperazino)phosphoroxy]-1-propenyl 2,3-(3,4,5-trimethoxybenzo)-butylphostonate (57)

Tetrabutylammonium fluoride (1.0 M solution in THF, 2 mmol) is added to a solution of silyl ether **56** (2 mmol) in 50 mL of THF cooled to -23° C. After 5 minutes, triethylamine (2 mmol) is added, followed by $POCl_3$ (2 mmol). After 4 hours, a mixture of N-trifluoroacetylpiperazine (2 mmol) and triethylamine (2 mmol) is added in one portion. After an additional 3 hours, NH_3 is added. After a further 2 hours, the reaction mixture is poured into ice-cold brine and extracted with ether (4 x 100 mL). The combined organic phases are dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. Purification by flash chromatography gave the product as a colorless oil.

Example 9: Prodrug activity of Galactosyl-cytosine Arabinoside

The prodrug galactosyl-cytosine arabinoside (GalAraC) has been prepared as outlined in example 3 and has been tested *in vitro* and *in vivo* for toxicity and activation by the bacterial enzyme, β-glactosidase.

Different concentrations of the prodrug GalAraC were added to the two different tissue cultures cell lines, Colo 320 DM and Lovo. The cells were grown for four days after which

the culture medium was removed and the cells were washed in PBS. After the cells were stained with Giemsa stain, the optical density of the stained cultures bound to the culture well surface was measured at 600 nm. The reduction of the optical density indicates a reduction of cell density adhering to the culture well. The same procedure was used to test the toxicity of AraC itself on the two cell lines. The comparison of the toxicity between prodrug and drug on the Colo 320 DM cell line is shown in Figure 9. By comparing the concentration of prodrug and drug at the concentrations used to give an OD (600) of 0.5 it can be seen that the GalAraC is at least 800-fold less toxic than the AraC. That is, one must use 800-fold higher concentration of GalAraC to achieve the equivalent toxicity as AraC itself. Similar results can be seen in Figure 10 using the cell line Lovo where the prodrug is again at least 800 times less toxic than the drug AraC. Both Figures 9 and 10 show that when the prodrug is activated by the enzyme β-galactosidase the toxicity is equivalent to that of the pure drug at the same concentration.

To test if the prodrug could be activated by β–glactosidase, the enzyme was conjugated to an antibody that was directed against Carcino Embryonic Antigen (CEA) a specific tumor antigen on the surface of the LoVo culture cells. The Colo 320 DM cells lack this surface antigen and were used as controls. The β-galactosidase conjugated antibody was added to the cultures and allowed to bind the antigen. The prodrug, at different concentrations, was then added to the cultures, which were then grown for three days. As controls, BSA without enzyme was added to the cultures and the same range of prodrug concentrations was added to the cultures. Figure 11 displays the results of this experiment which shows that the prodrug can be activated by the antibody-enzyme conjugate. By comparing Figure 11 with Figure 12, it is clear that the prodrug was not only activated by the conjugated antibody but also that the LoVo cells, carrying the CEA tumor marker are specifically killed when compared to the cultures where BSA was added with prodrug. The above results show that the prodrug is approximately 200-fold less toxic than the drug in an antigen localization experiment, and that

it can be activated by a bacterial enzyme specifically at the surface of a tumor cell when bound by antibody at the cell surface.

To assess the ability of surface-bound conjugate to generate cytotoxic levels of active drug, the rate of product formation was measured using ONPG as a substrate. Conjugate specifically bound to LoVo cells was found to generate 1.2×10^7 molecules of product/min/cell. In our particular assay format, this rate is equivalent to 1.6 mM product formed per minute. Since 1.5 mM AraC is reported to inhibit cellular proliferation by 50% (Gish, D., et al., J. Med. Chem. 14 (1971):1159) the experimentally obtained rate appears to be sufficient to generate cytotoxicity *in vitro* assay.

Both AraC and GalAraC have been tested for toxicity and activation in the mouse. In separate experiments, mice were given (at a concentration of 100 mg/kg), AraC, GalAraC and GalAraC followed by β-galactosidase. After five days, a complete blood count was made on all the mice. By comparing the drug with the prodrug (see bars in Figure 13), it is clear that the prodrug is substantially less toxic than the drug *in vivo*. Similarly, the data in Figures 14 to 17 (the key in Figure 13 is the same for Figures 14 to 17) show that in the presence of β-galactosidase, the prodrug can be activated to create a toxicity much like the drug itself. This effect is quite pronounced in segmented neutrophils and is less so in red blood cells which probably reflects the different kinetics of cell synthesis in the different cell populations.

In summary, these data show that the prodrug, GalAraC, has a significantly reduced toxicity *in vivo* and *in vitro* and that when activated by an enzyme, the activated prodrug is released creating very simliar toxicity as AraC *in vivo* and *in vitro*.

Example 10: Prodrug activity of Galactosyl-5-fluorouridine (24)

In a similar set of experiments the prodrug galactosyl-5-fluorouridine (Gal5FU) has been synthesized as described in Example 4 and tested as described above for GalAraC. The results of toxicity studies of the prodrug and the drug are shown in Figure 18. It can be seen that there is over a 500-fold increase in the concentration of the prodrug required to cause a similar degree of toxicity as the drug 5-Fluorouridine. As with GalAraC the prodrug gal5FU can be activated *in vitro* to produce levels of the drug similar to the pure drug itself. Thus, the addition of the galactose moiety onto the drug reduces the toxicity substantially and to a level that makes it an excellent candidate for a prodrug.

Galactosyl-5-fluorouridine has been tested for targeted activation by β-galactosidase conjugated to an antibody with the same CEA antigen tumor surface specificity as was done with the galAraC prodrug. The antibody was allowed to react with antigen-carrying cells (LoVo) and control cells without the CEA antigen marker. The prodrug was then added to different cultures at different concentrations.

Figure 19 displays the results of this experiment which shows that the antibody localized on the surface of the LoVo cells releases a toxic amount of 5-FU from the prodrug at a twenty- to thirty-fold lower concentration than in the control Colo cells (see Figure 20). Thus, site specific activation of the prodrug increases the efficacy of the drug at a significantly lower concentration.

Both gal5FU and 5FU have been tested and compared in mice. The *in vivo* studies were performed as described for the *in vivo* galAraC experiments. The drug and prodrug were administered and blood cell counts along with total bone marrow cellularity were measured 6 days following injection. The results of this experiment are displayed in Figure 21 through 25. The figure key in Figures 23 and 24 are the same as for Figure 21.

In a pattern similar to the results of the galAraC experiments, the prodrug showed reduced toxicity when compared to the drug itself. This is particularly evident in the neutrophil (see Figure 23) and lymphocyte (see Figure 24) cell populations. Total leukocytes (see Figure 21) show the same marked effect while the red cell population is not severly depleated in this 6 day experiment. The overall difference between the effect of the drug and the less toxic nature of the prodrug are most clearly seen in the measurements of total bone marrow cellularity. These results are displayed in Figure 25.

Not only is there no effect of the prodrug, it is also clear that it can be actived with β-galactosidase. Thus, the concept of galactosyl-AraC and galactosyl-5-fluoro-uracil to be used as prodrugs is not only a reasonable approach but from these data should stand a reasonable chance of success.

Example 15: Preparation of the Intermediate of the Prodrugs in Examples 16 and 20 and of the Haptens of the Prodrugs in Examples 18 and 22, the (thiazolyl)iminoacetic ester, Compound 60.

Refer to Figure 26 for the bold numbered compounds in this example.

The N-alkoxyphthalimide of bromide **58** is prepared, and then it is treated with hydrazine and 2-formamido-4-thiazolylglyoxylic acid to give acid **59**, following the procedure of Takasugi, et al., J. Antibiotics 36 (1983):846-854. Activation of the acid carboxyl using N-hydroxysuccinimide gives N-hydroxysuccinimidyl (Z)-2-(2-formamido-4-thiazolyl)-2-(1-*tert*-butoxycarbonyl-1-methyl)ethoxyiminoacetate **60**.

In detail, the synthesis is as follows:

tert-Butyl 2-bromo-2-methylpropanoate (**58**)

Isobutene is condensed into a solution of 2-bromo-2-methylpropanoic acid (10 mmol) and trifluoromethane sulfonic acid (0.1 mmol) in 100 mL of CH_2Cl_2 until the starting material is consumed, as observed by TLC. The volatile components are evaporated *in vacuo*, and the residue is filtered through a pad of neutral alumina using 50% ether/hexane. The filtrate is concentrated *in vacuo* and used without further purification.

(Z)-2-(2-Formamido-4-thiazolyl)-2-(1-*tert*-butoxycarbonyl-1-methyl)ethoxyiminoacetic acid (**59**)

Compound **59** is synthesized using bromide **58**, N-hydroxyphthalimide, and ethyl 2-(formylamino)-4-thiazoleglyoxylate following the procedure given by Takasugi, H., et al., J. Antibiotics 36 (1983):846-854, incorporated herein by reference.

N-Hydroxysuccinimidyl (Z)-2-(2-formamido-4-thiazolyl)-2-(1-*tert*-butoxycarbonyl-1-methyl)ethoxyiminoacetate (**60**)

A solution of DCC (11 mmol) in 10 mL of CH_2Cl_2 is added to a solution of N-hydroxysuccinimide (10 mmol) and acid **59** (10 mmol) in 90 mL of CH_2Cl_2 at room temperature. A precipitate forms quickly. After 1 hour, the solution is filtered and the filtrate is washed with water (40 mL), dried over anhydrous $MgSO_4$, and concentrated *in vacuo* to give the product as a colorless solid.

Example 16: Preparation of the Prodrug, the 5-fluorouridine substituted β-lactam, Compound 68.

Refer to Figure 27 for the bold numbered compounds in this example.

3-(S)-Amino-4-(S)-hydroxymethylazetidinone (**61**), prepared following the procedure of Evans, D. A., et al., Tetrahedron Lett. (1985):3783-3786, incorporated herein by reference, is acylated with ester **60** to give amide **62**, which then undergoes Swern oxidation and Baeyer-Villiger rearrangement (based on the method of Afonso, A., et al., in Bentley, et al., d. "Recent Advances in the Chemistry of β-Lactam Antibiotics" , The Royal Society of Chemistry Special Publ. No. 70 (1989):295-302, incorporated herein by reference) to give ester **64**. Protected 5-fluorouridine **65** is prepared and reacted with ester **64** to give azetidinone **66** (based on the method of Aoki, et al., Heterocycles 15 (1981):409-413 and Tetrahedron Lett. (1979):4327-4330; both incorporated herein by reference), where the alcohol is added to the azetidinone stereoselectively *trans* to the acylamino group. Azetidinone **66** is sulfonylated following the procedure of Cimarusti, C. M., et al., Tetrahedron 39 (1983):2577-2589, incorporated herein by reference, and deprotected to give prodrug **68**.

In detail, the synthesis is as follows:

3-(S)-Amino-4-(S)-hydroxymethylazetidineone (61)

Amine **61** can be made following the procedure of Evans, D. A., et al., Tetrahedron Lett. (1985):3783-3786, incorporated herein by reference.

3-(S)-[(Z)-2-(2-Formamido-4-thiazolyl)-2-(1-*tert*-butoxycarbonyl-1-methyl)ethoxyiminoacetyl]amino-4-(S)-hydroxymethylazetidinone (62)

Amine **61** (1 mmol), activated ester **60** (1 mmol), and DMAP (1 mmol) are dissolved in 10 mL of DMF. After the starting material is consumed as observed by TLC, the mixture is poured into water (50 mL) and extracted with ethyl acetate (3 x 50 mL), the organic phases are washed with brine (50 mL) and dried over anhydrous $MgSO_4$, and the solvent is

evaporated *in vacuo*. The residue is purified by flash chromatography to give the product as a colorless oil.

4-(R,S)-Carbonyl-3-(S)-[(Z)-2-(2-formamido-4-thiazolyl)-2-(1-*tert*-butoxycarbonyl-1-methyl)ethoxyiminoacetyl]aminoazetidinone (63)

A solution of oxalyl chloride (1.1 mmol) in 10 mL of CH_2Cl_2 is cooled to -78° C, and a solution of DMSO (1.1 mmol) in 1 mL of CH_2Cl_2 is added dropwise. After 15 minutes, a solution of alcohol **62** (1 mmol) in 1 mL of CH_2Cl_2 is added dropwise. After 30 minutes, a solution of triethylamine (1.2 mmol) in 1 mL of CH_2Cl_2 is added in one portion, and the mixture is allowed to warm to room temperature. The mixture is poured into water (50 mL) and extracted with CH_2Cl_2 (3 x 50 mL), the organic phases are washed with water (50 mL) and dried over anhydrous $MgSO_4$, and the solvent is evaporated *in vacuo*. The residue is purified by flash chromatography to give the product as a colorless oil.

4-(R,S)-Carbonyloxy-3-(S)-[(Z)-2-(2-formamido-4-thiazolyl)-2-(1-*tert*-butoxycarbonyl-1-methyl)ethoxyiminoacetyl]aminoazetidinone (64)

m-CPBA (1.5 mmol) is added to a solution of aldehyde **63** (1 mmol) in 10 mL of CH_2Cl_2 and the mixture is allowed to stand at room temperature until the starting material is consumed as observed by TLC. The mixture is poured into 1M $NaHCO_3$ (50 mL) and extracted with ethyl acetate (3 x 50 mL), the organic phases are dried over anhydrous $MgSO_4$, and the solvent is evaporated *in vacuo*. The residue is purified by flash chromatography to give the product as a colorless oil.

2',3'-O-Isopropylidene-5-fluorouridine (65)

2,2-Dimethoxypropane (2 mL) was added to a solution of 5-fluorouridine (1.05 g, 4 mmol) and TsOH (20 mg) in 5 mL of DMF. After the starting material was consumed as observed by TLC, 20 mL of methanol was added, and the reaction was allowed to stand overnight. Then the solvents were evaporated *in vacuo*. The resulting solid was recrystallized from hot methanol to give 864 mg of the product as a colorless solid.

^{1}H NMR (DMSO-d$_6$) d 1.26 (s, 3), 1.45 (s, 3), 3.50-3.66 (m, 2), 4.04-4.14 (m, 1), 4.70-4.78 (m, 1), 4.82-4.91 (m, 1), 5.81 (bs, 1), 8.17 (d, 1, J=7 Hz), 11.86 (bs, 1)

Preparation of the Prodrug Precursor (66)

A solution of $BF_3.OEt_2$ (0.1 mmol) in 1 mL of CH_2Cl_2 is added to a solution of ester **64** (1 mmol) and alcohol **65** (1 mmol) in 5 mL of CH_2Cl_2. After the starting material is consumed as observed by TLC, the mixture is poured into 0.1M $NaHCO_3$ (50 mL) and extracted with ethyl acetate (3 x 50 mL), the organic phases are dried over anhydrous $MgSO_4$, and the solvent is evaporated *in vacuo*. The residue is purified by flash chromatography to give the product as a colorless oil.

Preparation of the Prodrug Precursor 67

Trimethylsilyl chlorosulfonate (2 mmol) is added to DMF (4 mL). After 30 minutes, the volatile components are removed *in vacuo*. The residue is added to a mixture of amide **66** (1 mmol) in 4 mL of CH_2Cl_2 cooled by an ice bath. After 30 minutes, the solution is poured into 10 mL of 0.5M KH_2PO_4. The organic phase is separated, and the aqueous phase is extracted with CH_2Cl_2 (4 mL) and evaporated to dryness. The solid residue is triturated with methanol (40 mL), and the organic washings are concentrated *in vacuo*. The residue is used without further purification.

Preparation of the 5-fluorouridine-substituted β-lactam Prodrug (68)

Trifluoroacetic acid (1 mL) is added to a mixture of compound **67** (1 mmol) and anisole (0.5 mL) in 4 mL of CH_2Cl_2 cooled by an ice bath. The mixture is allowed to warm to room temperature, and after 1 hour, the volatile components are evaporated *in vacuo*. The residue is purified by reverse-phase HPLC using 0.1M triethylammonium acetate buffer (pH 7) and acetonitrile mixture as the mobile phase. The fractions containing the product are combined and dried *in vacuo*, the residue is redried from deionized water (2 x), and the residue is then passed through a SP-Sephadex ion exchange column, potassium form, to give the product as the dipotassium salt.

Example 17: Preparation of the Intermediate of the Hapten of the Prodrug in Example 16, the 5-alkynylated uridine, Compound 74.

Refer to Figure 28 for the bold numbered compounds in this example.

The hydroxyl groups of uridine **3a** are protected to give compound **70**. Compound **70** is iodinated in the 5 position to give iodide **71**. A subsequent palladium-catalyzed alkynylation gives compound **73**, which is selectively deprotected at the 5' hydroxyl to give the intermediate **74**.

In detail, the synthesis is as follows:

5-Iodo-2',3'-O-isopropylideneuridine (69)

A solution of triol **3a** (10 mmol, synthesis described in Example 16), 2,2-dimethoxypropane (30 mmol), and TsOH (1 mmol) in 10 mL of CH_2CL_2 is stirred at room temperature until the starting material is consumed as observed by TLC. Triethylamine (2 mmol) is added, and the

volatile components are evaporated *in vacuo*. The residue is purified using flash chromatography to give the product as a colorless solid.

5-Iodo-2',3'-O-isopropylidene-5'-O-(4-methylbenzoyl)uridine (70)

4-Methylbenzoyl chloride (10 mmol) is added to a solution of alcohol **69** (10 mmol) in 20 mL of pyridine. After no further progress occurs as observed by TLC, the volatile components are evaporated *in vacuo*. The residue is purified using flash chromatography to give the product as a colorless solid.

4-*tert*-Butoxycarbonylamino-1-butyne (72)

The crude amine 71, obtained after hydrazinolysis as described in Example 1b, is dissolved in 50 ml of dioxane and 2 mL of triethylamine, and a solution of di-*tert*-butyl dicarbonate (10 mmol) in 10 mL of dioxane is added. After the reaction is complete as observed by TLC, the mixture is partitioned between 0.05M HCl (50 mL) and ethyl acetate (3 x 100 mL), the organic phases are dried over $MgSO_4$, and the solvent is evaporated *in vacuo*. The residue is purified using flash chromatography to give the prduct as a colorless oil.

5-(4-*tert*-Butoxycarbonylamino-1-butynyl)-2',3'-O-isopropylidene-5'-O-(4-methylbenzoyl)uridine (73)

To a degassed solution of iodide **70** (5 mmol) in 150 mL of triethylamine is added 4-*tert*-butoxycarbonylamino-1-butyne **72** (10 mmol), $(Ph_3P)_2PdCl_2$ (0.2 mmol), and CuI (0.3 mmol). The resulting suspension is heated at 50° C until the starting material is consumed. The volatile components are evaporated *in vacuo*, and the residue is taken up in $CHCl_3$ (200 mL) and washed with 5% disodium EDTA (2 x 100 mL) and water (100 mL), dried over

$MgSO_4$, and the solvent is evaporated *in vacuo*. The residue is purified by flash chromatography to give the product as a colorless solid.

5-(4-*tert*-Butoxycarbonylamino-1-butynyl)-2',3'-O-isopropylideneuridine (**74**)

Concentrated ammonium hydroxide (7 mL) is added to a solution of ester **73** (5 mmol) in 90 mL of methanol. After the starting material is consumed as observed by TLC, the volatile components are evaporated *in vacuo* and the residue is purified by flash chromatography to give the product as a colorless oil.

Example 18: Preparation of the Hapten of the Prodrug in Example 16, the cyclobutanol substituted 5-fluorouridine, Compound 81.

Refer to Figures 29 and 30 for the bold numbered compounds in this example.

Alcohol **74** undergoes a conjugate addition to ethynylsulfonate **75** to give the enol ether **76**. Azidoketene undergoes a [2+2] cycloaddition to enol ether **76** to give cyclobutanone **77**. Reduction of the keto, azido, and alkynyl groups gives amino alcohol **79**, which is N-acylated and deprotected to give compound **81**, which can be linked to a carrier protein at the primary aliphatic amino group.

In detail, the synthesis is as follows:

tert-Butyl ethynsulfonate (**75**)

A solution of ethynylmagnesium chloride in THF (0.5M, 10 mmol) is added to a solution of sulfuryl chloride (20 mmol) in 100 mL of THF cooled to -78°C. After 1 hour, a solution of *tert*-butanol (60 mmol) and triethylamine (60 mmol) in 50 mL of THF is added dropwise.

The solution is allowed to warm to room temperature, the volatile components are evaporated *in vacuo*, the residue is partitioned between ether (150 mL) and 0.05M HCl (50 mL), the organic phase is washed with brine (50 mL) and dried over $MgSO_4$, and the volatile components are evaporated *in vacuo*. The residue is purified by flash chromatography to give the product as a colorless oil.

<u>Preparation of uridine 5'-O-enol ether **76**</u>

Sodium methoxide (0.05 mmol) is added to a solution of alkyne **75** (11 mmol) and alcohol **74** (10 mmol) in 100 mmol of THF. After the starting material is consumed as observed by TLC, acetic acid (0.1 mmol) is added, and the volatile components are evaporated *in vacuo*. The residue is partitioned between 5% $NaHCO_3$ (40 mL) and ethyl acetate (3 x 100 mL), the organic phases are dried over $MgSO_4$, and the solvent is evaporated *in vacuo*. The residue is purified by flash chromatography to give the product as a colorless oil.

<u>Preparation of Cyclobutanone **77**</u>

A solution of azidoacetyl chloride (10 mmol) in 20 mL of CH_2Cl_2 is added dropwise to a solution of triethylamine (11 mmol) and enol ether **76** (5 mmol) in 50 mL of CH_2Cl_2 cooled to -78° C. The mixture is allowed to warm slowly to room temperature overnight. When no further progress in the reaction is observed by TLC, 1 mL of methanol is added and the volatile components are evaporated *in vacuo*. The residue is passed through a short column of silica gel using ethyl acetate as a solvent.

<u>Preparation of cyclobutanol **78**</u>

Sodium borohydride (10 mmol) is added to a solution of ketone **77** (2 mmol) in 20 mmol of methanol cooled by an ice bath. When no further progress in the reaction is observed by

TLC, the volatile components are evaporated *in vacuo*. The residue is partitioned between 0.05M HCl (40 mL) and ethyl acetate (3 x 100 mL), the organic phases are dried over $MgSO_4$, and the solvent is evaporated *in vacuo*. The residue is then passed through a short column of silica gel using ethyl acetate as a solvent, concentrated *in vacuo*, and used without further purification.

Preparation of amino alcohol **79**

Azide **78** (5 mmol) is dissolved in methanol (100 mL), 5% Pd-C (10% by weight) is added, and the mixture is stirred under a hydrogen atmosphere until the starting material is consumed. The catalyst is filtered out using a pad of Celite, and the catalyst is rinsed with methanol (100 mL). The solvent is evaporated *in vacuo*, and the product is used without further purification.

Preparation of amide **80**

Amide **80** is synthesized from amine **79** and ester **60** following the procedure used for amide **62**.

Preparation of hapten **81**

Compound **80** is deprotected to give compound **81** using the procedure for compound **68**. However, the trifluoroacetate salt may be used for the reaction linking compound **81** at the primary aliphatic amino group to the carrier protein.

Example 19: Preparation of the Intermediate of the Prodrug in Example 20, the 5-fluorouridine 5'-O-aryl ester, Compound 85.

Refer to Figure 31 for the bold numbered compounds in this example.

Lithium-halogen exchange on bromide **5**, followed by reaction with benzyl chloroformate, gives monoester **82**. Esterification with 5-fluorouridine **65** gives diester **83**, which is selectively deprotected and activated at the benzyl ester group to give the intermediate **85**.

In detail, the synthesis is as follows:

2-Carbobenzyloxy-3,4,5-trimethoxybenzoic acid (**82**)

tert-Butyllithium (1.7M solution in n-pentane, 15 mmol) is added to a solution of 2-bromo-3,4,5-trimethoxybenzoic acid **5** (5 mmol, synthesis described in Example 12) in 50 mL of THF, while maintaining the temperature of the mixture below -95° C. After the addition is completed, the mixture is allowed to warm to -78° C. After 30 minutes, benzyl chloroformate (5 mmol) is added in one portion, and the mixture is allowed to warm to 0° C. Water (50 mL) is added, and then the pH of the mixture is carefully adjusted to 3 using 0.1 M HCl. The mixture is extracted with ethyl acetate (5 x 100 mL). The organic phases are dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The mixture is purified by flash chromatography to give the product as a colorless oil.

Preparation of diester **83**

A mixture of acid **82** (5 mmol), 2',3'-O-isopropylidene-5-fluorouridine **65** (5 mmol), and EDC (6 mmol) in 50 mL of CH_2Cl_2 is stirred at room temperature until the starting material is consumed. The solution is washed with water (2 x 30 mL), the aqueous phases are washed with CH_2Cl_2 (2 x 50 mL), the organic phases are dried over anhydrous $MgSO_4$, and the solvent is evaporated *in vacuo*. The residue is purified by flash chromatography to give the product as a colorless oil.

Preparation of monoacid 84

Diester **83** (2 mmol) is dissolved in ethyl acetate (100 mL), 5% Pd-C (10% by weight) is added, and the mixture is stirred under a hydrogen atmosphere until the starting material is consumed. The catalyst is filtered out using a pad of Celite, and the catalyst is rinsed with ethyl acetate (100 mL). The solvent is evaporated *in vacuo*, and the product is used without further purification.

Preparation of acid chloride 85

Monoacid **84** (1 mmol) is dissolved in CH_2Cl_2 (20 mL), and thionyl chloride (5 mmol) is added. The progress of the reaction is monitored by methanolysis of aliquots and ^{1}H-NMR spectroscopy. When the reaction is complete, the volatile components are evaporated *in vacuo* to give compound **85** as an oil.

Example 20: Preparation of the Prodrug, the β-lactam substituted by a 5'-O-aroyl-5-fluorouridine, Compound 90.

Refer to Figure 32 for the bold numbered compounds in this Example.

N-acylation and amidation, using hydroxylamine, of threonine gives hydroxamic acid **87**. Reaction of compound **87** at the more acidic hydroxamic acid hydroxyl using acid chloride **85** gives amide **88** (Miller, M. J., et al. Tetrahedron 39 (1983):2575), which undergoes ring closure by a Mitsunobu reaction (Miller, M. J., et al., J. Am. Chem. Soc. 102 (1980):7026-7032). Subsequent deprotection gives the β-lactam prodrug **90**.

In detail, the synthesis is as follows:

N-[(Z)-2-(2-Formamido-4-thiazolyl)-2-(1-*tert*-butoxycarbonyl-1-methyl)ethoxyiminoacetyl]threonine (86)

A mixture of L-threonine (5 mmol), ester **60** (5 mmol), and DMAP (5 mmol) in 30 mL of DMF is stirred at room temperature. After the starting material is consumed as observed by TLC, the mixture is poured into 0.05M HCl (50 mL) and extracted with ethyl acetate (3 x 50 mL), the organic phases are washed with brine (50 mL) and dried over anhydrous $MgSO_4$, and the solvent is evaporated *in vacuo*. The residue is purified by flash chromatography to give the product as a colorless oil.

Preparation of threonine hydroxamic acid 87

A solution of DCC (5.5 mmol) in 5 mL of CH_2Cl_2 is added to a solution of hydroxylamine hydrochloride (5 mmol), triethylamine (5 mmol), and acid **86** (5 mmol) in 45 mL of CH_2Cl_2 at room temperature. A precipitate forms quickly. After 1 hour, the solution is filtered and the filtrate is washed with 0.05M HCl (40 mL), the aqueous phase is extracted with CH_2Cl_2 (50 mL), and the organic phases are dried over anhydrous $MgSO_4$, and concentrated *in vacuo* to give the product as a colorless solid.

Preparation of O-benzoyl hydroxamic acid 88

Compound **85** is taken up in 5 mL of CH_2Cl_2 and added dropwise to a solution of hydroxamic acid **87** (1 mmol) and DMAP (2 mmol) in 20 mL of CH_2Cl_2 cooled by an ice bath. After 2 hours, the mixture is poured into water (50 mL) and extracted with ethyl acetate (3 x 50 mL), the organic phases are washed with brine (50 mL) and dried over anhydrous $MgSO_4$, and the solvent is evaporated *in vacuo*. The residue is purified by flash chromatography to give the product as a colorless oil.

Preparation of β-lactam **89**

A solution of DEAD (1.1 mmol) in 10 mL of THF is added dropwise to a solution of compound **88** (1 mmol) and triphenylphosphine (1.1 mmol) in 20 mL of THF at room temperature. After the reaction is complete as observed by TLC, the solvent is evaporated *in vacuo*. The residue is purified by flash chromatography to give the product as a colorless oil.

Preparation of β-lactam prodrug **90**

Trifluoroacetic acid (2 mL) was added to a mixture of compound **89** (1 mmol) and anisole (1mL) in 10 mL of CH_2Cl_2 cooled by an ice bath. The mixture is warmed to room temperature, and, after 1 hour, the volatile components are evaporated *in vacuo*. The residue is purified by reverse-phase HPLC using 0.1M triethylammonium acetate buffer (pH 7) and acetonitrile mixture as the mobile phase. The fractions containing the product are combined and dried *in vacuo*, the residue is redried from deionized water (3 x), and the residue is then passed through a SP-Sephadex ion exchange column, potassium form, to give the product as the potassium salt.

Example 21: Preparation of the Intermediate of the Hapten in Example 22, the 5-alkynylated uridine 5'-O-aryl ester, Compound 92.

Refer to figure 33 for the bold numbered compounds in this Example.

Esterification of acid **82** using alcohol **74** gives diester **91**. Selective deprotection of the benzyl ester carboxyl group gives the monoacid **92**.

In detail, the synthesis is as follows:

Synthesis of adriamycin prodrug **103** can be prepared starting from adriamycin **101** and benzoic acid **102** (Figure 35). Adriamycin **101** is treated with benzoic acid **102** in the presence of 1-ethyl 3-(3-dimethylaminopropyl) carbodiimide (EDC) and 1-hydroxybenzotriazole (HOBT) in DMF. In detail, the synthesis is as follows:

General procedure for the synthesis of Benzoylamide of Adriamycin Prodrug 103.

To a solution of Adriamycin **101** (1 eq) in DMF (0.015 M) add sequentially the benzoic acid **102** (1eq), 1-ethyl 3(3 dimethylaminopropyl)carbodiimide (EDC, 1.05 eq) and 1-hydroxy benzotriazole (HOBT 1 eq) and stir the reaction mixture under argon atmosphere at room temperature. After completion of the reaction purify the product **103** by chromatography. Other aroylamides can be prepared using the same procedure by substituting the appropriate aroyl carboxylic acids.

Example 24: Preparation of the hapten of the prodrug in Example 23, the phosphate of the aroylamide of adriamycin compound 104.

Refer to Figure 36 for the bold numbered compounds in this example.

The synthesis of transition state analog of adriamycin, compound **104**, can be prepared starting from adriamycin **101** and benzenephosphonic acid **105**. Adriamycin **101** is treated with benzenephosphonic acid **105** in the presence of EDC and 1-hydroxybenzotriazole in DMF.

In detail, the synthesis is as follows:

To a solution of adriamycin **101** (1 eq) in DMF add sequentially the benzenephosphonic acid **105** (1 eq), 1-ethyl 3(3 dimethylaminopropyl) carbodiimide (EDC, 1 eq) and 1-hydroxybenzotriazole (1 eq) and stir the reaction mixture at room temperature. After completion of the reaction, the product **104** can be purified by chromatography.

Example 24a: Preparation of the hapten of the prodrug in Example 23, the aroyl sulphonamides of adriamycin compound 106:

Refer to Figure 37 for the bold numbered compounds in this example.

Aroyl sulphonamide hapten compound **106** can be prepared by treating adriamycin **101** with benzenesulfonyl chloride **107** in the presence of triethylamine in dry DMF.

In detail, the synthesis is as follows:

Synthesis of TS analog compound 106

To a solution of adriamycin **101** (1 eq) in DMF in the presence of triethylamine (1.5 eq) under argon atmosphere is added slowly at 0° C benzenesulphonyl chloride **107** (1.1 eq). The reaction mixture is stirred at room temperature and after completion of the reaction, the product **106** can be purified by chromatography.

Example 25: Preparation of melphalan aroylamide prodrugs 109

Refer to Figure 38 for the bold numbered compounds in this example.

Synthesis of melphalan prodrug **109** could be accomplished starting from melphalan **108** and benzoyl chloride.

Detail synthesis of compound **109** follows the same procedure as described for the preparation of **106** where benzoyl chloride is used instead of benzenesulphonyl chloride.

Example 26a: Preparation of the hapten of the prodrug in example 25, the sulphonamide compound 110

Refer to Figure 39 for the bold numbered compounds in this example.

Synthesis of hapten of melphalan, compound **110**, could be achieved starting from the melphalan **108** and benzenesulphonyl chloride **107** using the similar reaction conditions as described for the preparation **106**.
Detail synthesis of the compound follows the same procedure as described for the synthesis of **106**.

Example 27: Relative toxicities of 5-fluorouridine ester prodrugs

Fluorouridine is a cytotoxic antineoplastic nucleoside analog, with clinical utility in treating solid tumors in various tissues. Fluorouridine is, however, toxic to normal tissues, particularly bone marrow and gastrointestinal epithelium. Prodrugs of fluorouridine that are activated by catalytic antibodies or enzymes targeted to tumor cells improve the therapeutic index of fluorouridine substantially. It is preferred that the prodrugs not be activated by endogenous enzymes, but rather are readily activated by catalytic antibodies.

Catalytic antibodies which cleave esters are prepared through straight-forward methods. Ester substituents attached to the 5'-position of fluorouridine render it non-toxic and protect it from degradation by uridine phosphorylase. 5'-benzoate and substituted 5'-benzoate prodrugs of fluorouridine were administered to mice to determine whether substituents on the

benzoate moiety could modify deesterification by endogenous enzymes thereby resulting in prodrugs that are substantially less toxic than fluorouridine itself.

Methods

Fluorouridine (FUrd) and fluorouridine prodrugs were administered to groups (n=7) of 20-gram female Balb/c mice by intraperitoneal injection, in the following doses:

1. Fluorouridine 10 mg/kg.
2. Fluorouridine 50 mg/kg.
3. Fluorouridine 100 mg/kg.
4. 5'-Benzoylfluorouridine (BZ-FUrd) 139.7 mg/kg.
5. 5'-(2,4,6-trimethylbenzoyl)fluorouridine (TMB-FUrd) 156.9 mg/kg.
6. 5'-(3,4,5-trimethoxybenzoyl)fluorouridine (TMOX-FUrd) 175.2 mg/kg.

The doses of the three aromatic esters of fluorouridine are the molar equivalent of 100 mg/kg fluorouridine.

A seventh group (Control) received only the injection vehicle (0.4 ml of 10% DMSO in 0.9% saline).

Seven days after administration of fluorouridine or its prodrugs, blood samples were taken from the retro-orbital sinus for determination of differential blood cell counts, cells from one femur of each mouse were collected for counting total marrow cellularity, and spleens were collected and weighed. Body weight was also determined.

Results

Fluorouracil administration resulted in dose-dependent reductions in blood cell counts and marrow cell counts.

100 mg/kg fluorouridine produced a significant reduction in body weight and spleen weight. 5'-Benzoylfluorouridine (139 mg/kg), which was expected to be cleaved by mouse esterase activity was approximately equal in toxicity to a molar equivalent of fluorouridine alone (100 mg/kg), as is reflected in all indices tested.

5'-(2,4,6-trimethylbenzoyl)fluorouridine (TMB-FUrd) produced very little evidence of toxicity, with only erythrocyte counts significantly below control values. This compound produced less damage to bone marrow, as determined by marrow cell count and neutrophil counts, than did 1/10 the molar equivalent of fluorouridine (FUrd 10 mg/kg).

5'-(3,4,5-trimethoxybenzoyl)fluorouridine (TMOX-FUrd) was slightly less toxic than 1/2 the molar equivalent of fluorouridine (FJ 50 mg/kg).

Data are shown in Tables 1 and 2.

Table 1:

Cellularity Groups	Body Weight (grams)	Spleen Weight (mg)	Marrow 10^6 cells/femur)
Control	20.1±0.5	89.9±3.4	8.28±0.69
FUrd 10 mg/kg		89.9±2.0 ns	5.83±0.77 *
FUrd 50 mg/kg		69.6±2.4 *	2.85±0.16 *
FUrd 100 mg/kg	16.3±0.6 *	57.7±2.5 *	0.98±0.19 *
BZ-FUrd	17.5±0.6 *	61.8±1.2 *	1.23±0.10 *
TMB-FUrd	19.6±0.4 ns	99.2±4.4 ns	7.88±0.47ns
TMOX-FUrd	20.0±0.5 ns	73.3±3.5 *	3.42±0.29 *

Legend:* indicates significantly lower than Control value, P < .05; ns indicates not different from Control (untreated) group.

Table 2: Relative toxicities of fluorouridine and fluorouridine prodrugs--blood cell counts.

Groups	Platelets (K/ml)	Neutrophils (K/ml)	Erythrocytes (K/ml)
Control	741±15	1.747±.737	9.01±0.09
FUrd 10 mg/kg	705±14 ns	607±.330 ns	8.33±0.09 *
FUrd 50 mg/kg	433±39 *	.020±.036 *	7.81±0.11 *
FUrd 100 mg/kg	155±20 *	.010±.019 *	7.59±0.25 *
BZ-FUrd	209±31 *	.011±.030 *	7.76±0.22 *
TMB-FUrd	707±23 ns	1.30±.338 ns	8.69±0.07 *
TMOX-FUrd	628±27 *	.093±.039 *	7.65±0.11 *

Legend:* indicates significantly lower than Control value, $P < .05$; ns indicates not different from Control (untreated) group.

Example 27a: Relative Toxicities of 5-Fluorouridine Ester Prodrugs at High Doses

In experiments similar to those described in Example 27, the toxicity of 2,4,6 trimethoxybenzoyl 5-fluorouridine and 2,6 dimethoxybenzoyl 5-fluorouridine were tested in mice at high dose levels in an attempt to determine the maximum tolerated dose.

Part I.

2,4,6 trimethoxybenzoyl 5-fluorouridine was compared with the toxicity of 5-fluorouridine and controls in 6 groups of Balb C female mice as listed below:

1)	Control - Saline	0.2ml i.p.	5 animals
2)	FlUrd	10 mg/kg	5 animals
3)	FlUrd	50 mg/kg	5 animals
4)	Trimethoxybenzoyl FlUrd (TMOXFlUrd)	158 mg/kg	
	(molar equivalent of 100 mg/kg FlUrd)		5 animals
5)	TMOXFlUrd	316 mg/kg	
	(molar equivalent of 200 mg/kg FlUrd)		5 animals
6)	TMOXFlUrd	790 mg/kg	
	(molar equivalent of 500 mg/kg FlUrd)		3 animals

Seven days after administration of the drugs, blood samples were taken from the retro orbital sinus for determination of differential blood counts.

Results

As Table 3 shows below the prodrug modified blood cell counts at the highest dosage only, equivalent to 500 mg/kg of the drug. The toxicity shown at this dose was approximately equivalent to a dose of slightly more than 10 mg/kg of the FlUrd drug itself indicating a toxicity ratio of about 50:1. These high doses of prodrug did not kill the animals proving that they have a very substantial reduction in their toxicity when compared to the FlUrd drug.

Table 3: Effect Of FlUrd Versus 2,4,6 Trimethoxybenzoyl FlUrd

Group	WBC Lymphocytes K/µl	Platelets K/µl	Neutrophils K/µl	K/µl
Control	10.32±0.34	730.6±33.7	1.635±0.259	8.31±0.2
FlUrd 10 mg/kg	9.82±0.89 ns	683.8±25.6 ns	0.654±0.200*	8.79±0.7
ns				
FlUrd 50 mg/kg	12.03±0.77 ns	312.3±45.3*	0.058±0.033*	11.90±0.76
TMOX FlUrd 100 mg/kg	10.22±0.80 ns	730.6±58.7 ns	1.674±0.212 ns	7.91±0.6
ns				
TMOX FlUrd 200 mg/kg	9.28±0.32 ns	833.8±79.6 ns	0.985±0.167 ns	7.83±0.1
ns				
TMOX FlUrd 500 mg/kg	7.23±0.24*	771.7±29.5 ns	0.513±0.231*	6.60±0.2

Legend: * indicates significantly lower than Control value, P<.05; ns indicates not different from Control (untreated) group

Part II.

High doses of 2,6 dimethoxybenzoyl 5-fluorouridine was compared with the toxicity of 5-fluorouridine and controls in 8 groups of Balb C female mice as listed below.

1) Control - Saline 0.2ml i.p. 7 animals
2) FlUrd 5 mg/kg 6 animals
3) FlUrd 10 mg/kg 6 animals

4) FlUrd 50 mg/kg 6 animals

5) FlUrd 100 mg/kg 6 animals

6) 2,6 Dimethoxybenzoyl FlUrd (DMOX FlUrd) 6 animals
   the molar equivalent of 100 mg/kg FlUrd

7) DMOX FlUrd, the molar equivalent of 200 mg/kg FlUrd 6 animals

8) DMOX FlUrd, the molar equivalent of 500 mg/kg FlUrd 5 animals

Seven days after administration of the drugs, blood samples were taken from the retro orbital sinus for determination of differential blood counts.

Results

As shown in Table 4 below, 2,6 dimethylbenzoyl fluorouridine in the large doses used in this experiment produced no evidence of toxicity as measured by leukocytes, platelets, neutrophils and lymphocytes. This prodrug especially at these high doses is very non-toxic, with a toxicity ratio relative to FlUrd of greater than 50:1 for neutrophils, the blood cell type most sensitive to cytotoxic chemotherapy drugs.

Table 4: Effects Of FlUrd Versus 2,6 Dimethoxy FlUrd On Blood Cell Counts

Group	WBC K/μl	Platelets K/μl	Neutrophils K/μl	Lymphocytes K/μl
Control	7.34±0.46	769.1±26.4	0.971±0.141	6.02±0.33
FlUrd 5 mg/kg	7.43±0.55 ns	736.0±37.2 ns	1.473±0.386 ns	5.58±0.38
ns				
FlUrd 10 mg/kg	8.27±0.64 ns	820.5±34.6 ns	0.637±0.084 ns	7.16±0.59
ns				
FlUrd 50 mg/kg	4.88±0.70*	489.0±72.3*	0.208±0.183*	4.62±0.51*
FlUrd 100 mg/kg	1.88±0.46*	178.3±14.8*	0.007±0.003*	1.87±0.46*
DMOX FlUrd 100 mg/kg	7.48±0.29	784.3±11.9	1.335±0.101	5.86±0.37
DMOX FlUrd 200 mg/kg	9.90±0.76	895.0±25.9	2.083±0.242	7.43±0.76
DMOX FlUrd 500 mg/kg	9.02±0.59	909.6±30.3	1.972±0.194	6.78±0.60

Legend: * indicates significantly lower than Control value, P<.05; ns indicates not different from Control (untreated) group

Example 28: Relative toxicities of 5-fluorouridine and 5'-β-galactosyl-fluorouridine

5'-β-galactosyl-fluorouridine (Gal-Furd) is a prodrug of fluorouridine which can be activated by the non-mammalian enzyme β-galactosidase or by an appropriate catalytic antibody. A crucial issue is the degree to which a sugar attached covalently to the 5' position reduces the toxicity of fluorouridine. The primary dose-limiting toxicity for antineoplastic fluorinated pyrimidine analogs is damage to bone marrow. The toxicity of fluorouridine versus Gal-Furd was assessed in mice, using blood cell counts and bone marrow cell counts as the indices of

toxicity. In addition, Gal-Furd was administered together with the enzyme β-galactosidase to determine if the prodrug could be activated by an enzyme *in vivo*.

Female Balb/C mice (20 grams) were divided into 6 groups, each containing 6 animals:

1. Control - Saline 0.2 ml i.p.
2. Fluorouridine - 10 mg/kg i.p.
3. Fluorouridine - 100 mg/kg i.p.
4. Gal-Furd - 160 mg/kg i.p. (molar equivalent of 100 mg/kg fluorouridine)
5. β-galactosidase - 5 mg/kg i.p.
6. Gal-Furd 160 mg/kg + β-galactosidase 5 mg/kg i.p. (Gal-Furd was administered after β-galactosidase in a separate injection).

Seven days after administration of fluorouridine or Gal-Furd, blood samples were taken from the retro-orbital sinus for determination of differential blood cell counts, and cells from one femur of each mouse were collected for counting total marrow cellularity; spleens were also collected for determination of their weight.

Results

Seven days after administration of fluorouridine resulted in significant declines in all hematologic indices tested. In contrast, blood cell and bone marrow cell counts seven days after administration of Gal-Furd were within the range of normal values for Balb/C mice. Coadministration of Gal-Furd and β-galactosidase (each administered by a separate injection so that prodrug and enzyme were not in contact prior to administration) resulted in hematologic toxicity, indicating that the relatively nontoxic prodrug was converted to active cytotoxic drug by the enzyme β-galactosidae *in vivo*. The results are summarized in Tables 5 and 6, and in Figure 25.

Table 5: Effects of Furd versus Gal-Furd on spleen weight and marrow cellularity

Groups	Marrow Cellularity Spleen Wt (mg)	(10^6 cells/femur)
Control	92.8±3.5	8.86±1.09
FUrd 10 mg/kg	100.5 ns	------------
FUrd 100 mg/kg	53.5±2.1 *	0.96±0.25 *
Gal-Furd 160 mg/kg	89.9±3.4 ns	9.70±0.81 ns
Galactosidase	91.3±1.9 ns	-----------
Gal-Furd + Galactosidase	80.2±4.3 *	4.04±0.84 *

Legend:* indicates significantly lower than Control value, P < .05; ns indicates not different from Control (untreated) group.

Table 6: Effects of Furd versus Gal-Furd on blood cell counts

Groups	Platelets (k/ml)	Neutrophils (k/ml)	Lymphocytes (M/ml)
Control	833±30	2.25±.22	10.37±0.68
FUrd 10 mg/kg	809±28 ns	0.75±.15 *	7.28±0.67 *
FUrd 100 mg/kg	242±12 *	0.08±.02 *	3.07±0.23 *
Gal-Furd 160 mg/kg	770±25 ns	1.90±.22 nd	7.39±0.45 *
Gal-Furd+Galactosidase	572+39 *	0.74+.07 *	4.78+o.21 *

Legend* indicates significantly lower than Control value, P < .05; ns indicates not different from Control (untreated) group.

Example 28a Relative Toxicity of 5-Fluorouridine and 5'-ß-Galactosyl Fluorouridine at High Levels

In a similar experiment to the one described in Example 28, larger doses of 5'-ß-galactosyl fluorouridine was tested to determine the toxic limits of the prodrug. Female Balb C mice were divided and treated with single i.p. doses of the drugs as follows:

1) FlUrd	150 mg/kg	5 animals
2) FlUrd	200 mg/kg	5 animals
3) FlUrd	250 mg/kg	5 animals
4) Galactosyl FlUrd	750 mg/kg	3 animals
5) Galactosyl FlUrd	1500 mg/kg	3 animals

The mice were checked daily for mortality and signs of toxicity. Seven days after administration of fluorouridine, blood samples were taken from 2 animals in each group.

Results

The results of this experiment are shown in Table 7 below. Beginning about 5 days after drug treatment, all of the mice that received fluorouridine began to look scruffy and lost about 20% of their body weight. The mice that received galactosylfluorouridine showed no overt signs of toxicity at any time.

Neutrophil counts are the most sensitive indicators of bone marrow damage caused by fluorouridine. Gal-FlUrd at 1500 mg/kg changed neutrophil counts less than did fluorouridine at a dose of 10 mg/kg. Neutrophil counts after 1500 mg/kg Gal-FlUrd are in fact within the normal range (1-2.5K cells/microliter). The toxicity ratio of Gal-FlUrd to FlUrd toward bone marrow *in vivo* is greater than 100:1, i.e. FlUrd is more than 100 times as toxic toward marrow as is Gal-FlUrd.

Gal-FlUrd at a dose of 1500 mg/kg is essentially non-toxic in Balb C mice. This dose is far higher than would be administered in a therapeutic scenario involving targeted activation of fluorouridine prodrugs by anti-linked catalytic proteins.

Table 7: Effects of FlUrd Versus High Doses Of Gal-FlUrd On Mortality And Blood Cell Counts

A. Mortality

Group	Mortality
FlUrd 150 mg/kg	2/5
FlUrd 200 mg/kg	5/5
FlUrd 250 mg/kg	5/5
Gal-FlUrd 750 mg/kg	0/3
Gal-FlUrd 1500 mg/kg	0/3

No animals died until 10 days after treatment with fluorouridine. All animals that died did so between 10 and 15 days after drug administration. The published LD50 for 5-fluorouridine in mice is 160 mg/kg, which corresponds well with the mortality results obtained as a function of dose of fluorouridine in this study.

B. Blood Cell Counts

Group	WBC K/μl	Neutrophils K/μl	Platelets K/μl
FlUrd 150 mg/kg	1.0	0.015	145.5
FlUrd 200 mg/kg	0.8	0.02	115

FlUrd 250 mg/kg	0.75	0.01	98
Gal-FlUrd 750 mg/kg	7.25	1.705	862
Gal-FlUrd 1500 mg/kg	6.6	1.315	835

Since only 2 animals from each group were sampled for blood cell counts, average values are given with no statistics.

Example 29: Relative toxicities of Cyclophosphamide and Aldophosphamide diethylacetal

Cyclophosphamide is an antineoplastic alkylating agent that must undergo enzymatic conversion in the liver to form precursors of its active cytotoxic catabolites. Thus, although cyclophosphamide is a clinically important drug, it is not itself a suitable candidate for targeted delivery. Its active cytotoxic catabolite precursors, e.g., aldophosphamide are unstable. The diethyl acetal of aldophosphamide was prepared as a prodrug of aldophosphamide which could be activated in a single catalytic step by a suitable catalytic protein, such as catalytic antibody. In this experiment, cyclophosphamide and aldophosphamide diethyl acetal were administered to mice to determine whether aldophosphamide diethyl acetal would in fact be relatively non-toxic and therefore suitable for targeted activation by an antibody-catalyst conjugate.

Female Balb/C mice (20 grams) were divided into 4 groups, each containing 7 animals:

1. Control - Saline 0.2 ml i.p.
2. Cyclophosphamide (CYP) - 30 mg/kg i.p.
3. Cyclophosphamide - 150 mg/kg i.p.
4. Aldophosphamide diethyl acetal (ALP-DEA) - 188 mg/kg i.p. (molar equivalent of 150 mg/kg cyclophosphamide).

Four days after administration of the drugs, blood samples were taken from the retro-orbital sinus for determination of differential blood cell counts, and cells from one femur of each mouse were collected for counting total marrow cellularity.

Results

Four days after administration of cyclophosphamide (150 mg/kg), there were significant declines in all hematologic indices tested. In contrast, leukocyte and bone marrow cell counts four days after administration of aldophosphamide diethyl acetal were within the range of normal values for Balb/C mice. The aldophosphamide diethyl acetal in fact did not reduce neutrophil counts, which were significantly reduced by the lower dose of cyclophosphamide (30 mg/kg). Neutrophil count is perhaps the most sensitivity index for hematopoietic damage caused by the active catabolites of cyclophosphamide. Thus, aldophosphamide diethyl acetal is relative non-toxic to hematopoietic cells compared to cyclophosphamide.

Table 8: Effects of cyclophosphamide versus aldophosphamide diethyl acetal on marrow cellularity.

Groups	Marrow Cellularity (10^6 cells/femur)
Control	6.33±0.45
CYP 30 mg/kg	6.03±0.29 ns
CYP 150 mg/kg	2.09±0.14 *
ALP-DEA 188 mg/kg	7.79±0.59 ns

Note: * indicates significantly lower than Control values, $P < .05$; ns indicates not different from Control (untreated) group.

Table 9: Effects of cyclophosphomide versus aldophosphamide diethyl acetal on blood cell counts

Groups	Neutrophils (K/μl)	Lymphocytes (M/μl)	Platelets (K/μl)
Control	1.11±.10	5.59±0.44	775±25
CYP 30 mg/kg	0.47±.08	4.14±0.19 *	796±20 ns
CYP 150 mg/kg	0.04±.01 *	1.98±0.12 *	591±14 *
ALD-DEA 188 mg/kg	1.20±.18 ns	5.52±0.40 ns	743±12 ns

Note: * indicates significantly lower than Control values, $P < .05$; ns indicates not different from Control (untreated) group.

Example 30: Relative Toxicities of Melphalan, Benzoyl Melphalan and 3,4,5 Trimethoxybenzoyl Melphalan

Melphalan is the phenylalanine derivative of nitrogen mustard also known as L-sarcolysin. This alkylating agent is frequently used to treat multiple myeloma, carcinoma of the breast and ovary and some beneficial effects have been reported for malignant melanoma. The toxicity of melphalan is mostly hematological and is similar to that of other alkylating agents.

Benzoyl and 3,4,5 trimethoxybenzoyl melphalan were prepared as prodrugs of melphalan which were designed to be activated by a single step catalytic antibody cleavage to release the active drug, melphalan.

In this experiment, the hematological toxicities of the prodrugs were compared with the active drug. The prodrugs were administered in amounts equivalent to 5, 10 and 20 mg/kg of melphalan. Balb C females were divided into 10 groups of 6 animals which received drugs and dosages listed below:

1) Control - Saline 0.2 ml i.p.

2)	Melphalan (Mel)	5mg/kg i.p.
3)	Melphalan	10 mg/kg i.p.
4)	Melphalan	20 mg/kg i.p.
5)	Benzoyl melphalan (B Mel)	(molar equivalent of 5mg/kg of melphalan)
6)	B Mel	(molar equivalent of 10 mg/kg of mel)
7)	B Mel	(molar equivalent of 20 mg/kg of mel)
8)	3,4,5 trimethoxybenzoyl melphalan (TMB)	(molar equivalent of 5 mg/kg mel)
9)	TMB	(molar equivalent to 10 mg/kg of mel)
10)	TMB	(molar equivalent to 20 mg/kg of mel)

Four days after administration of the drugs, blood samples were taken from the retro orbital sinus for determination of differential blood counts.

Results

As shown in the Table 10 below, four days after administration of melphalan, there were significant declines in all the hematologic indices tested. In contrast, following the administration of the prodrug, the leukocyte counts were either not significantly changed (in some cases slightly elevated) or when slightly depressed the largest dose of prodrug never depressed the counts to the level near the lowest doses of melphalan. Neutrophil counts were not changed from normal with either prodrug at any dosage. Thus, the two prodrugs show significant reduction in their toxicity over melphalan.

Table 10: Effects Of Melphalan Versus Benzoyl Melphalan And Trimethoxybenzoyl Melphalan On Blood Cell Counts

Group	WBC K/µl	Platelets K/µl	Neutrophils K/µl	Lymphocytes K/µl
Control	7.34±0.46	769.1±26.4	0.971±0.141	6.02±0.33
Mel 5 mg/kg	2.66±0.21*	779.6±42.6 ns	0.354±0.036*	2.18±0.19*
Mel 10 mg/kg	1.28±0.09*	643.0±28.9*	0.078±0.011*	1.17±0.07*
Mel 20 mg/kg	0.06±0.08*	570.0±41.5*	0.026±0.007*	0.58±0.08*
B Mel 5 mg/kg	5.34±0.49*	746.3±17.5 ns	0.980±0.105 ns	4.09±0.48*
B Mel 10 mg/kg	5.34±0.31*	869.6±20.1	1.141±0.157 ns	3.98±0.26*
B Mel 20 mg/kg	5.49±0.37*	881.9±12.8	1.357±0.190 ns	3.76±0.20*
TMB mel 5 mg/kg	6.88±0.36 ns	681.2±26.2*	1.126±0.178 ns	5.38±0.46 ns
TMB mel 10 mg/kg	4.67±0.28*	723.2±23.0 ns	0.995±0.110 ns	3.45±0.18*
TMB mel 20 mg/kg	5.54±0.41*	755.4±26.4 ns	0.928±0.099 ns	4.44±0.48*

Legend: * indicates significantly lower than Control value, P<.05; ns indicates not different from Control (untreated) group

Example 31: Preparation of the Prodrug, tetrakis(2-chloroethyl)aldophosphamide diethyl acetal, Compound 112.

Refer to Figure 40 for the bold numbered compounds in this Example.

Phosphoramidic dichloride **36** was reacted with bis(2-chloroethyl)amine to form the phosphoramidic chloride **111**, which was then reacted with the lithium alkoxide of 3,3-diethoxy-1-propanol to form the prodrug **112**.

In detail, the synthesis is as follows:

N,N,N',N',-Tetrakis(2-chloroethyl)phosphorodiamidic chloride **111**

Triethylamine (1.18 mL, 8.4 mmol) was added to a mixture of dichloridate **36** (1.0 g, 3.9 mmol), bis(2-chloroethyl)amine hydrochloride (0.758 g, 4.2 mmol) and 38 mL of toluene at room temperature. The mixture was then heated at reflux for 16 hours. After cooling to room temperature, the mixture was washed with 10% KH_2PO_4 (2 x 20 mL), the aqueous phases were extracted with ether (2 x 10 mL), and the combined organic phases were concentrated and purified by flash chromatography (25% ethyl acetate/hexane, product R_f 0.25 in 30% ethyl acetate/hexane) to give 0.52 g of an oil (37%); ^1H NMR ($CDCl_3$) d 3.45-3.63 (m, 8), 3.65-3.78 (m, 8).

Synthesis of Compound **112**

A 2.5 M solution of n-BuLi in hexane (0.81 mL, 2.0 mmol) was added to a solution of 3,3-diethoxy-1-propanol (0.19 mL, 1.3 mmol) in 6 mL of THF at room temperature. After 30 minutes, the mixture was cooled to 0° C, and chloridate **111** (0.47 g, 1.3 mmol) was added. The mixture was allowed to warm to room temperature. After 1 hour, a solution of 10% NaH_2PO_4 (8 mL) was added, and the mixture was extracted with ether (3 x 8 mL), the organic phases were dried over anhydrous $MgSO_4$, and the solvent was evaporated *in vacuo*. The residue was purified by flash chromatography (eluting with 25, 30, 40, and 50% ethyl acetate/hexane, product R_f 0.22 in 30% ethyl acetate/hexane) to give 132 mg of the product as an oil (21%); IR (neat) 2975, 2932, 2899, 2879, 1455, 1375, 1347, 1306, 1225, 1132,

1088, 1056, 980, 921, 893, 760, 723, 658 cm^{-1}; ^{1}H NMR ($CDCl_3$) d 1.22 (t, 6, J=7.0 Hz), 2.00 (q, 2, J=6.1 Hz), 3.36-3.70 (m, 20), 4.11 (q, 2, J=6.4 Hz), 4.63 (t, 1, J=5.6 Hz); ^{13}C NMR ($CDCl_3$) d 15.30, 34.72, 34.82, 42.31, 49.65, 49.71, 61.70, 62.20, 99.83.

Example 32: Preparation of the Hapten of the Prodrug in Example 31: The trimethylammonium salt analog of tetrakis(2-chloroethyl)aldophosphamide diethyl acetal, Compound 119.

Refer to Figure 41 for the bold numbered compounds in this Example.

A linker moiety was first prepared, and then attached to the phosphorus of the hapten. The nitrogen of glycine was protected as the *p*-nitrobenzyl urethane to form compound **113**. The carboxyl group was then activated as the N-hydroxysuccinimide ester, forming compound **114**, which was reacted with excess piperazine to form the linker moiety, compound **115**. Compound **115** was reacted with the dichloridate **36** to form the monochloridate **116**. Compound **116** was reacted with the lithium alkoxide of 2-(dimethylamino)ethanol, giving the phosphorodiamide **117**. The tertiary amine of compound **117** was quaternized using MeI to give compound **118**. Attempts to deprotect the analog of compound **118**, where the glycine was protected as the less reactive benzyl urethane, failed; however, the more labile *p*-nitrobenzyl urethane protecting group was readily removed to give the hapten **119**.

<u>In detail</u>, the synthesis is as follows:

<u>Synthesis of Compound **113**</u>

A solution of 4-nitrobenzyl chloroformate (3.16 g, 14.6 mmol) in 15 mL of dioxane was added to a solution of glycine (1.0 g, 13.3 mmol) in 7 mL of water, maintaining the pH of the solution at 9 using triethylamine. The mixture was allowed to stir for 65 hours. The mixture

was then washed with ether, the pH of the aqueous phase was adjusted to 1, and the mixture was extracted with ethyl acetate, the organic phase was dried over anhydrous $MgSO_4$, and the solvent was evaporated *in vacuo* to give 3.9 g of the product as an oil; ^1H NMR ($CDCl_3$) d 4.05 (d, 2, J=6 Hz), 5.23 (s, 2), 5.43 (d, 1), 7.52 (d, 2, J=8 Hz), 8.22 (d, 2, J=8 Hz).

Synthesis of Compound **114**

Pyridine (1.24 mL, 15.4 mmol) and N,N'-disuccinimidyl carbonate (3.93 g, 15.3 mmol) were added to a mixture of acid **113** (3.9 g, 15.3 mmol) and 76 mL of acetonitrile at room temperature. After 16 hours, the solvent was evaporated *in vacuo*, the residue was dissolved in ethyl acetate and washed with water, the organic phase was dried over anhydrous $MgSO_4$, and the solvent was evaporated *in vacuo* to give 4.41 g of the product as an oil (82%).

Synthesis of Compound **115**

A solution of compound **114** (4.4 g, 12 mmol) in 400 mL of CH_2Cl_2 was added dropwise to a rapidly stirred mixture of piperazine (5.4 g, 63 mmol) and 1000 mL of CH_2Cl_2 cooled to -78° C. The mixture was allowed to warm to room temperature overnight. The mixture was concentrated to a volume of 200 mL and extracted with 5% HCl, the pH of the aqueous layer was adjusted to 9 using Na_2CO_3, and the aqueous layer was extracted with ethyl acetate and CH_2Cl_2. The organic phases were dried over anhydrous Na_2SO_4, the solvent was evaporated *in vacuo*, and the product was purified by flash chromatography (10% methanol/CH_2Cl_2, product R_f 0.19) to give 1.50 g of an oil (37%); ^1H NMR ($CDCl_3$) d 2.84 (bs, 4), 3.36 (bs, 2), 3.58 (bs, 2), 4.01 (bs, 2), 5.20 (bs, 2), 6.00 (bs, 1), 7.49 (d, 2, J=8 Hz), 8.17 (d, 2, J=8 Hz).

Synthesis of Compound **116**

Triethylamine (0.24 mL, 1.7 mmol) was added to a mixture of amine **115** (0.55 g, 1.7 mmol) and 9 mL of toluene. Then dichloridate **36** (0.44 g, 1.7 mmol) was added, and the mixture was heated at reflux for 14 hours, during which time some dark, insoluble material formed. The mixture was cooled, poured into saturated NaH_2PO_4, and extracted with ethyl acetate and CH_2Cl_2. The organic phases were dried over anhydrous Na_2SO_4, the solvent was evaporated *in vacuo*, and the product was purified by flash chromatography (90% ethyl acetate/hexane, product R_f 0.65 in ethyl acetate) to give 0.2 g of an oil (22%); ^1H NMR ($CDCl_3$) d 3.23-3.40 (m, 4), 3.40-3.63 (m, 6), 3.63-3.84 (m, 6), 4.00-4.07 (m, 2), 5.23 (s, 2), 5.87 (s, 1), 7.53 (d, 2, J=8 Hz), 8.22 (d, 2, J=8 Hz).

Synthesis of Compound **117**

A 2.5 M solution of n-BuLi in hexane (0.154 mL, 0.39 mmol) was added to a solution of 2-(dimethylamino)ethanol (37 mL, 0.37 mmol) in 1.5 mL of THF at 0° C. The mixture was allowed to stir at room temperature for 1 hour. The solution was cooled again to 0° C, and a solution of chloridate **116** (0.2 g, 0.37 mmol) in 2.5 mL of THF was added. The mixture was allowed to stir for 1.5 hours at room temperature. Approximately 100 mL of triethylamine was then added to the mixture, the volatile components were evaporated *in vacuo*, and the product was purified by flash chromatography (5% methanol/CH_2Cl_2, product R_f 0.44 in 10% methanol/CH_2Cl_2). The product was dissolved in ethyl acetate and washed with 5% $NaHCO_3$. After drying the organic layer with anhydrous Na_2SO_4, the solvent was evaporated to give 0.1 g of the product as an oil (46%); ^1H NMR ($CDCl_3$) d 2.25 (s, 6), 2.54 (t, 2, J=5 Hz), 3.08-3.23 (m, 4), 3.28-3.45 (m, 6), 3.52-3.69 (m, 6), 3.95-4.01 (m, 2), 4.01-4.14 (m, 2), 5.18 (s, 2), 5.95 (s, 1), 7.47 (d, 2, J=8 Hz), 8.16 (d, 2, J=8 Hz).

Synthesis of Compound **118**

Methyl iodide (30 mL, 0.48 mmol) was added to a solution of amine **117** (100 mg, 0.16 mmol) in 2 mL of THF at room temperature. A yellow insoluble oil formed over 24 hours. The volatile components were evaporated *in vacuo* to give 125 mg of a yellow oil; IR (CD_3OD) 2952, 2855, 1709, 1651, 1607, 1522, 1453, 1412, 1372, 1350, 1277, 1235, 1220, 753, 725 cm^{-1}; ^1H NMR (CD_3OD) d 3.27 (s, 9), 3.19-3.61 (m, 12), 3.71 (dd, 4, J=6.3, 6.3 Hz), 3.82 (bs, 2), 4.03 (s, 2), 4.50 (bs, 2), 5.23 (s, 2), 7.60 (d, 2, J=8.2 Hz), 8.21 (d, 2, J=8.2 Hz).

Synthesis of Compound **119**

Compound **118** (124.6 mg, 0.17 mmol) was dissolved in 6 mL of 1:1 methanol and water, 10% Pd-C (12 mg) was added, and the mixture was stirred under a hydrogen atmosphere for 18 hours. The mixture was filtered through a pad of Celite, washing with 1:1 methanol and water, and the volatile components were removed *in vacuo* to give 89 mg of a yellow solid (94%); ^1H NMR (CD_3OD) d 3.29 (s, 9), 3.16-3.30 (m, 4), 3.38-3.56 (m, 6), 3.56-3.68 (m, 4), 3.68-3.79 (m, 4), 3.82-3.90 (m, 2), 4.50 (bs, 2).

Example 33: Preparation of the Hapten of the Prodrug in Example 31: The dipropylmethylammonium salt analog of tetrakis(2-chloroethyl)aldophosphamide diethyl acetal, Compound 121.

Refer to Figure 42 for the bold numbered compounds in this Example.

2-(Di-n-propylamino)ethanol, compound **120**, is prepared following the procedure of W. W. Hartmann in Organic Syntheses, Collective Vol. II; Blatt, A. H., Ed.; John Wiley & Sons: New York, (1943):183-184, incorporated herein by reference. Compound **120** is reacted with compound **116**, and the product is transformed in two additional steps to give the hapten **121**.

In detail, the synthesis is as follows:

2-(Di-n-propylamino)ethanol **120**

Compound **120** is synthesized following the procedure of Hartman, W. W. In Organic Syntheses, Collective Vol. II; Blatt, A. H., Ed.; John Wiley & Sons: New York, (1943):183-184, incorporated herein by reference, using dipropylamine and 2-chloroethanol.

Synthesis of Compound **121**

Compound **121** is synthesized from compounds **116** and **120** following the procedure used for the synthesis of compound **119** from compound **116** and 2-(diethylamino)ethanol (see Example 32).

Example 34: Preparation of the Prodrug, intramolecular bis(2-hydroxyethoxy)benzoate-5-fluorouridine, Compound 128.

Refer to Figure 43 for the bold numbered compounds in this Example.

2-Bromoethanol was protected as the *p*-methoxybenzyl ether to give compound **122**. Compound **123** was formed by condensing 2,6-dihydroxybenzoic acid and methanol. Compound **123** was dialkylated using bromide **122** to give compound **124**. In order to determine the stability of the prodrug **128** to undesired noncatalyzed lactonization and concommitant release of the drug, compound **124** was deprotected to form compound **125**. Compound **125** was dissolved in 0.9% NaCl in D_2O. No change was observed in the ^1H NMR spectrum of this sample after standing at room temperature for 96 hours. Compound

124 was saponified to give acid **126**, which was condensed with compound **65** to give compound **127**. Acidic deprotection of compound **127** gives the prodrug **128**.

In detail, the synthesis is as follows:

2-(4-Methoxybenzyloxy)bromoethane **122**

Trifluoromethanesulfonic acid (30 mL) was added to a mixture of 2-bromoethanol (0.5 mL, 6.7 mmol), 4-methoxybenzyl trichloroacetimidate (3.8 g, 13.4 mmol), and 15 mL of THF at room temperature. After 1 hour, the reaction was neutralized by the addition of 5% $NaHCO_3$, and the mixture was extracted with ethyl acetate. The organic layer was dried over anhydrous $MgSO_4$, concentrated, and the residue was purified by flash chromatography (eluting with 0, 1, and 2.5% ethyl acetate/hexane, product R_f 0.48 in 10% ethyl acetate/hexane) to give 1.3 g of an oil (79%); ^1H NMR ($CDCl_3$) d 3.49 (t, 2, J=7 Hz), 3.79 (t, 2, J=7 Hz), 3.82 (s, 3), 4.55 (s, 2), 6.91 (d, 2, J=9 Hz), 7.21 (d, 2, J=9 Hz).

Methyl 2,6-dihydroxybenzoate **123**

DCC (26.3 g, 127 mmol) was added to a mixture of 2,6-dihydroxybenzoic acid (10 g, 64 mmol) and 200 mL of a 1:1 mixture of methanol and CH_2Cl_2, and the mixture was stirred at room temperature for 64 hours. Then the insoluble material was removed by filtration, the resulting solution was concentrated *in vacuo*, the residue was dissolved in ethyl acetate and refiltered, the filtrate was washed with water and brine, dried over anhydrous $MgSO_4$, concentrated, and the residue was purified by flash chromatography (10% ethyl acetate/hexane, product R_f 0.29) to give 8.14 g of a colorless solid (76%); ^1H NMR ($CDCl_3$) d 4.08 (s, 3), 6.48 (d, 2, J=8 Hz), 7.31 (dd, 1, J=8, 8 Hz).

Methyl 2,6-bis[2-(4-methoxybenzyloxy)ethoxy]benzoate **124**

A mixture of diphenol **123** (50 mg, 0.30 mmol), bromide **122** (292 mg, 1.19 mmol), K_2CO_3 (414 mg, 3.0 mmol), and 6 mL of DMF was stirred for 6 hours at room temperature. An additional quantity of K_2CO_3 was then added. After an additional 17 hours, the mixture was heated at 80° C for 1 hour. After cooling, the pH of the mixture was adjusted to 5 by the addition of 1 M HCl. The mixture was partitioned between ethyl acetate and water, the organic layer was dried over anhydrous $MgSO_4$, the solvent was evaporated *in vacuo*, and the residue was purified by flash chromatography (30% ethyl acetate/hexane, product R_f 0.58 in 50% ethyl acetate/hexane) to give 87 mg of the product as an oil (59%); ^1H NMR ($CDCl_3$) d 3.79-3.90 (m, 4), 3.81 (s, 6), 3.85 (s, 3), 4.20 (dd, 4, J=5, 5 Hz), 4.59 (s, 4), 6.59 (d, 2, J=9 Hz), 6.93 (d, 4, J=9 Hz), 7.27 (dd, 1, J=9, 9 Hz), 7.31 (d, 4, J=9 Hz).

Methyl 2,6-bis(2-hydroxyethoxy)benzoate **125**

To a solution of compound **124** (87 mg, 0.18 mmol) in 3 mL of methanol was added 8.7 mg of 10% Pd-C, and the mixture was stirred under a hydrogen atmosphere at room temperature for 1 hour. The catalyst was removed by filtration through Celite, washing with methanol. The solvent was evaporated *in vacuo*, and the residue was purified by preparative TLC (product R_f 0.54 in 70% ethyl acetate/hexane) to give 31 mg of the product as an oil (79%); ^1H NMR (CDCl3) d 3.82-3.96 (m, 4), 3.92 (s, 3), 4.08-4.20 (m, 4), 6.58 (d, 2, J=8 Hz), 7.29 (dd, 1, J=8, 8 Hz); (0.9% NaCl in D_2O) d 3.90 (dd, 4, J=4, 4 Hz), 3.97 (s, 3), 4.17 (dd, 4, J=4, 4 Hz), 6.79 (d, 2, J=8 Hz), 7.45 (dd, 1, J=8, 8 Hz).

Stability of Diol Ester **125** to Lactonization

A sample of diol ester **125** was dissolved in 0.9% NaCl in D_2O. No change was observed in the ^1H NMR spectrum of this sample after standing at room temperature for 96 hours.

2,6-Di[2-(4-methoxybenzyloxy)ethoxy]benzoic acid (126)

1 N NaOH (25 mL) was added to a mixture of compound 124 (1.22 g, 2.46 mmol) and 30 mL of dioxane, and then 10 mL of MeOH was added to the mixture to help maintain a homogeneous solution. The mixture was heated by an oil bath at 100° C for 24 hours. The mixture was cooled to room temperature and the pH of the solution was adjusted to 5 using 1 N HCl. The mixture was poured into ethyl acetate, washed with water and brine, and the organic phase was dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. The crude product, 1.1 g, was used without further purification; R_f 0.40 (5% MeOH/CH_2Cl_2); ^{1}H NMR ($CDCl_3$) d 3.76-3.89 (m, 10), 4.19 (dd, 4, J=9, 9 Hz), 4.55 (s, 4), 6.59 (d, 2, J=8 Hz), 6.88 (d, 4, J=8 Hz), 7.24-7.37 (m, 5).

Synthesis of Compound **127**

Compound 65 (81 mg, 0.27 mmol) was added to a mixture of compound 126 (516 mg, 1.07 mmol), 864 mL of pyridine, and 1 mL of CH_2Cl_2, and then EDC (205 mg, 1.07 mmol) and DMAP (65 mg, 0.53 mmol) were added. The mixture was heated at 80° C for 24 hours. The mixture was cooled to room temperature, and 10 mL of MeOH was added. After an additional 30 minutes, the volatile components were evaporated *in vacuo*, and the residue was taken up in ethyl acetate and washed with saturated $NaHCO_3$, water, saturated NH_4Cl and water, and the organic phase was dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. Purification of the residue by flash chromatography (eluting with 20, 30, 40, 50, and 60% ethyl acetate/hexane) gave 154 mg of the product (75%); R_f 0.50 (60% ethyl acetate in hexane); ^{1}H NMR ($CDCl_3$) d 1.34 (s, 3), 1.57 (s, 3), 3.71-3.76 (m, 4), 3.79 (s, 6), 4.16 (dd, 4), 4.29 (dd, 1, J=2.5, 12.2 Hz), 4.46 (d, 1, J=3.1 Hz), 4.46 (d, 2, J=12.6 Hz), 4.50 (d, 2, J=12.6 Hz), 4.66-4.74 (m, 2), 4.82 (dd, 1, J=3.2, 6.1 Hz), 5.90-5.91 (m, 1), 6.57

(d, 2, J=8.5 Hz), 6.86 (d, 4, J=8.6 Hz), 7.24 (d, 4, J=8.6 Hz), 7.28 (d, 1, J=8.5 Hz), 7.42 (d, 1, J=6.2 Hz), 9.11 (d, 1, J=4.2 Hz).

Synthesis of Compound **128**

The reaction is carried out following the procedure for the synthesis of compound **1a**.

Example 35: Preparation of the Hapten of the Prodrug in Example 34: The cyclic phosphonate analog of bis(2-hydroxyethoxy)benzoate-5-fluorouridine, Compound 137.

Refer to Figure 44 for the bold numbered compounds in this Example.

Resorcinol is monoalkylated using bromide **122** to give compound **129**. Phosphorylation of phenol **129** gives the phosphate triester **130**, which undergoes phosphorus migration after ortho-lithiation using LDA to give compound **131**. Hydroxyethylation of compound **131** by ethylene carbonate or glycol sulfite gives compound **132**, which is cyclized under high dilution conditions to give compound **133**. Saponification gives acid **134**, which is activated and reacted with compound **3f** to give compound **135**. The toluoyl groups of compound **135** are cleaved off to give compound **136**, which is deprotected and reduced to give the hapten **137**.

In detail, the synthesis is as follows:

Synthesis of Compound **129**

A mixture of resorcinol (5 mmol), compound **122** (1 mmol), K_2CO_3 (5 mmol), and 25 mL of DMF is stirred at room temperature until the starting material is consumed, as observed by

TLC. The mixture is neutralized with 0.1 M HCl, diluted with water, and extracted with ethyl acetate. The organic phase is dried over anhydrous $MgSO_4$ and concentrated, and the residue is purified by flash chromatography to give the product as a colorless oil.

Synthesis of Compound **130**

A mixture of diphenyl chlorophosphate (1.2 mmol) and 5 mL of CH_2Cl_2 is added to a mixture of compound **129** (1 mmol) and 5 mL of pyridine cooled to 0° C. After the starting material is consumed, as observed by TLC, the volatile components are evaporated *in vacuo*, and the residue is partitioned between ethyl acetate and 0.1 M HCl, the organic phase is dried over anhydrous $MgSO_4$ and concentrated, and the residue is purified by flash chromatography to give the product as a colorless oil.

Synthesis of Compound **131**

A 1.5 M solution of LDA in THF (1.1 mmol) is added dropwise to a solution of compound **130** (1 mmol) in THF (20 mL) cooled to -78° C. After the starting material is consumed, as observed by TLC, the mixture is partitioned between ethyl acetate and 0.1 M HCl, the organic phase is dried over anhydrous $MgSO_4$ and concentrated, and the residue is purified by flash chromatography to give the product as a colorless oil.

Synthesis of Compound **132**

A mixture of compound **131** (1 mmol), ethylene carbonate or glycol sulfite (10 mmol), K_2CO_3 (10 mmol), and 50 mL of DMF is heated at 100° C until the starting material is consumed, as observed by TLC. The mixture is cooled to room temperature, neutralized with 0.1 M HCl, diluted with water, and extracted with ethyl acetate. The organic phase is dried

over anhydrous $MgSO_4$ and concentrated, and the residue is purified by flash chromatography to give the product as a colorless oil.

Synthesis of Compound **133**

A mixture of compound **132** (1 mmol), anhydrous KF (10 mmol), 18-crown-6 (1 mmol), and 100 mL of THF is heated at reflux until the starting material is consumed, as observed by TLC. Then, the solvent is evaporated *in vacuo*, and the residue is purified by flash chromatography to give the product as a colorless oil.

Synthesis of Compound **134**

0.2 M NaOH (5 mL) was added to a solution of compound **133** (1 mmol) in 5 mL of dioxane at room temperature. After the starting material is consumed, as observed by TLC, the pH of the mixture is adjusted to 2 with 0.1 M HCl, and the mixture is extracted with ethyl acetate. The organic phase is dried over anhydrous $MgSO_4$, concentrated, and purified by flash chromatography to give the product as a colorless solid.

Synthesis of Compound **135**

A mixture of compound **134** (1.1 mmol) and 10 mL of thionyl chloride is stirred at room temperature until conversion to the acid chloride is complete, as determined by ^1H NMR of an aliquot of the reaction quenched with methanol. The unreacted thionyl chloride is evaporated *in vacuo*. The residue is taken up in 5 mL of CH_2Cl_2 and added slowly to a mixture of compound **3f** (1 mmol) and 5 mL of pyridine cooled to 0° C. After the starting material is consumed, as observed by TLC, the volatile components are evaporated *in vacuo*, the residue is taken up in ethyl acetate, the organic phase is washed with saturated $NaHCO_3$, 0.1 M HCl,

and brine, dried over anhydrous $MgSO_4$, and concentrated *in vacuo*. Purification of the residue by flash chromatography gives the product as a colorless solid.

Synthesis of Compound **136**

Concentrated ammonium hydroxide (1 mL) is added to a mixture of compound **135** (1 mmol) and 20 mL of methanol at 0° C. The solution is allowed to warm to room temperature. After the starting material is consumed, as observed by TLC, the volatile components are evaporated *in vacuo*, and the residue is purified by flash chromatography to give the product as a colorless solid.

Synthesis of Compound **137**

Ten percent Pd-C (10 weight %) is added to a mixture of compound **136** (1 mmol) and 20% aqueous methanol (10 mL), and the mixture is stirred under a hydrogen atmosphere. When the reaction is complete, the catalyst is removed by filtration through a pad of Celite, washing with 20% aqueous methanol. Evaporation of the volatile components *in vacuo* gives the product as a solid.

Example 36: Preparation of the Prodrug, intramolecular bis(3-hydroxypropyloxy)benzoate-5-fluorouridine, compound 138.

Refer to Figure 45 for the bold numbered compounds in this Example.

3-Bromo-1-propanol is transformed into the prodrug **138** using the same reactions as is used for the preparation of prodrug **128** (see Example 34).

In detail, the synthesis is as follows:

Synthesis of Compound **138**

Compound **138** is synthesized following the procedure for the synthesis of compound **128**, but starting with 3-bromo-1-propanol in place of 2-bromoethanol.

Example 37: Preparation of the Hapten of the Prodrug in Example 36: the cyclic phosphonate analog of bis(3-hydroxypropyloxy)benzoate-5-fluorouridine, Compound 139.

Refer to Figure 46 for the bold numbered compounds in this Example.

Resorcinol is transformed into the cyclic phosphonate hapten **139** using the same sequence of reactions used to prepare hapten **137** (see Example 35), using 3-bromopropyl 4-methoxybenzyl ether (prepared as an intermediate in Example 36).

In detail, the synthesis is as follows:

Synthesis of Compound **139**

Compound **139** is synthesized following the procedure for the synthesis of compound **137**, but starting with 3-bromo-1-propanol in place of 2-bromoethanol.

Example 38: Preparation of the Prodrug: 5'-O-(2,4,6-Trimethoxybenzoyl)-5-fluorouridine, Compound 141.

Refer to Figure 47 for the bold numbered compounds in this Example.

2,4,6-Trimethoxybenzoic acid was condensed with compound **65** using EDC to give ester **140**. Subsequently, the isopropylidene protecting group was removed to give the prodrug **141**.

In detail, the synthesis is as follows:

2',3'-O-Isopropylidene-5'-O-(2,4,6-trimethoxybenzoyl)-5-fluorouridine **140**

2,4,6-Trimethoxybenzoic acid (300 mg, 1.42 mmol) was dissolved in 2 mL of CH_2Cl_2, and 1.15 mL of pyridine was added. Compound **65** (106 mg, 0.35 mmol) was added, followed by EDC (300 mg, 1.56 mmol). The mixture was stirred for 24 hours before adding 10 mL of methanol. After an additional 30 minutes, the volatile components were evaporated *in vacuo*, and the residue was taken up in ethyl acetate (75 mL) and washed with saturated $NaHCO_3$ (2 x 50 mL), water (15 mL), saturated NH_4Cl (2 x 30 mL), and water (15 mL). All the aqueous phases were extracted with ethyl acetate (50 mL), and the organic phases were dried over anhydrous $MgSO_4$ and concentrated. The residue was purified by preparative TLC (10% methanol/CH_2Cl_2, R_f 0.50 in 8% methanol/CH_2Cl_2) to give 100 mg of the product as a colorless solid (58%); ^1H NMR ($CDCl_3$) d 1.38 (s, 3), 1.61 (s, 3), 3.81 (s, 6), 3.83 (s, 3), 4.33 (dd, 1, J=2.4, 12.3 Hz), 4.62 (dd, 1, J=small, 2.2 Hz), 4.72-4.77 (m, 2), 4.83 (dd, 1, J=2.1, 6.1 Hz), 5.92-5.93 (m, 1), 6.11 (s, 2), 7.58 (d, 1, J=6.3 Hz).

5'-O-(2,4,6-Trimethoxybenzoyl)-5-fluorouridine **141**

A mixture of compound **140** (100 mg, 0.20 mmol) and 1.5 mL of 50% formic acid was heated at 65° C for 2 hours. The mixture was cooled, and the volatile components were evaporated *in vacuo*. The residue was purified by preparative TLC (10% methanol/CH_2Cl_2, R_f 0.52) to give 84 mg of the product as a colorless solid (92%); ^1H NMR (CD_3OD) d 3.76 (s, 6), 3.80 (s, 3), 4.10-4.12 (m, 1), 4.18 (dd, 1, J=5.1, 5.1 Hz), 4.23-4.27 (m, 1), 4.34

(dd, 1, J=2.6, 16 Hz), 4.62 (dd, 1, J=2.2, 16), 5.88 (dd, 1, J=1.6, 4.1 Hz), 6.21 (s, 2), 7.76 (d, 1, J=6.6 Hz).

Example 39: Preparation of the Hapten of the Prodrug in Example 38, the pyridinium alcohol-substituted analog of uridine, compound 149.

Refer to Figures 48a and 48b for the bold numbered compounds in this Example.

Compound **142**, the aldehyde of compound **65**, is synthesized following a literature procedure. The aldehyde group undergoes a Wittig reaction to form compound **143**. Compound **144** was synthesized following literature precedent, and was brominated to give monobromide **145**. It was found that compound **144** must be used in an excess, and the reaction done at a low temperature, in order to get selective monobromination, otherwise the major product is 2,6-dibromo-3,4-dimethoxypyridine. Compound **145** undergoes lithium-halogen exchange, and the reactive intermediate is reacted with compound **143** to give the pyridinium alcohol **146**. Ammonolysis gives triol **147**, which is selectively methylated at the more nucleophilic pyridine nitrogen to give the quaternary ammonium salt **148**. Finally, reduction gives the hapten **149**.

In detail, the synthesis is as follows:

Synthesis of Compound **142**

Compound **142** is synthesized from compound **65** following the procedure in Ranganathan, R. S.; Jones, G. H.; Moffat, J. G. J. Org. Chem. 39 (1974):290-298, incorporated herein by reference.

Synthesis of Compound **143**

A solution of (triphenylphosphoranylidene)acetaldehyde (1.1 mmol) in 5 mL of CH_2Cl_2 is added to a solution of compound **142** (1 mmol) in 5 mL of CH_2Cl_2 at room temperature. After the starting material is consumed, as observed by TLC, the mixture is concentrated to half its volume and purified by flash chromatography to give the product as a colorless solid.

2-Bromo-3,5-dimethoxypyridine **145**

A solution of bromine (0.17 mL, 3.3 mmol) in 66 mL of CH_2Cl_2 was added dropwise to a solution of 3,5-dimethoxypyridine, compound **144**, (1.83 g, 13.2 mmol, prepared following the procedure of Johnson, C. D.; Katritzky, A. R.; Viney, M. J. Chem. Soc. (B), (1967):1211-1213, incorporated herein by reference in 66 mL of CH_2Cl_2 cooled to -78° C. After 1 hour, the mixture was allowed to warm slowly to room temperature over 16 hours. The volatile components were evaporated *in vacuo*, the residue was partitioned between ethyl acetate and aqueous sodium thiosulfate adjusted to pH 10 using 1 M NaOH, and the organic phase was dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The yellow oil was separated by flash chromatography (20% ethyl acetate/hexane) to give 1.0 g of the product (R_f 0.53 in 50% ethyl acetate/hexane) and 1.1 g of recovered starting material (R_f 0.28 in 50% ethyl acetate/hexane); ^1H NMR ($CDCl_3$) d 3.91 (s, 3), 3.93 (s, 3), 6.77 (bs, 1), 7.73 (bs,1).

Synthesis of Compound **146**

A 2.5 M solution of n-BuLi in hexane (0.4 mL, 1 mmol) is added to a solution of compound **145** (0.9 mmol) in 10 mL of THF at 0° C. After 1 hour, the mixture is cooled to -78° C, and a solution of compound **146** (0.9 mmol) in 1.5 mL of THF is added in one portion. The solution is allowed to warm to room temperature after 1 hour. After the starting material is consumed, as observed by TLC, water is added and the mixture is extracted with ethyl

acetate, the organic phase is dried over anhydrous Na_2SO_4 and concentrated *in vacuo*, and the residue is purified by flash chromatography to give the product as a colorless solid.

Synthesis of Compound **147**

Concentrated ammonium hydroxide (1 mL) is added to a mixture of compound **146** (1 mmol) and 20 mL of methanol at 0° C. The solution is allowed to warm to room temperature. After the starting material is consumed, as observed by TLC, the volatile components are evaporated *in vacuo*, and the residue is purified by flash chromatography to give the product as a colorless solid.

Synthesis of Compound **148**

A mixture of compound **147** (1 mmol), methyl iodide (2 mmol), and 10 mL of THF in a sealed tube is heated at 60° C until the starting material is consumed, as observed by TLC. The precipitate is filtered and washed with ether to give the product as a solid.

Synthesis of Compound **149**

Ten percent Pd-C (10 weight %) is added to a mixture of compound **148** (1 mmol) and 20% aqueous methanol (10 mL), and the mixture is stirred under a hydrogen atmosphere. When the reaction is complete, the catalyst is removed by filtration through a pad of Celite, washing with 20% aqueous methanol. Evaporation of the volatile components *in vacuo* gives the product as a solid.

Example 40: Relative toxicities of Cyclophosphamide and 3,3-diethoxypropyl N,N,N',N'-tetrakis(2-chloroethyl)phosphorodiamide (Tetrakis)

In this experiment, cyclophosphamide and 3,3-diethoxypropyl N,N,N',N'-tetrakis(2-chloroethyl)phosphorodiamide Tetrakis were administered to mice to determine whether aldophosphamide diethylacetal would in fact be relatively non-toxic, and therefore, suitable for targeted activation by an antibody-catalyst conjugate.

Female Balb/C mice (20 grams) were divided into 4 groups, each containing 5 animals:

1. Control - Saline 0.2 ml i.p.
2. Cyclophosphamide (CYP) - 30 mg/kg i.p.
3. Cyclophosphamide - 150 mg/kg i.p.
4. Tetrakis - 248 mg/kg i.p. (molar equivalent of 150 mg/kg cyclophosphamide

Five days after administration of the drugs, blood samples were taken from the retro-orbital sinus for determination of differential blood cell counts.

Results

Five days after administration of cyclophosphamide (150 mg/kg), there were significant declines in all hematologic indices tested. In contrast, leukocyte and bone marrow cell counts five days after administration of Tetrakis were within the range of normal values for Balb/C mice. The Tetrakis, in fact, did not reduce neutrophil counts, which were significantly reduced by the lower dose of cyclophosphamide (30 mg/kg). Neutrophil count is perhaps the most sensitivity index for hematopoietic damage caused by the active catabolites of cyclophosphamide. Thus, Tetrakis is relatively non-toxic to hematopoietic cells compared to cyclophosphamide.

Table 11: Effects of cyclophosphamide versus Tetrakis on blood cell counts.

Groups	Neutrophils (K/_l)	Lymphocytes (K/_l)	Platelets (K/_l)
Control	0.82+.32	4.26+0.68	765+41
CYP 30 mg/kg	0.41+.23 *	3.96+0.66 ns	776+37 ns
CYP 150 mg/kg	0.07+.06 *	2.53+0.26 *	867+109 ns
Tetrakis 248 mg/kg	0.775+.28 ns	3.45+0.88 ns	1000+177 ns

- indicates significantly lower than Control values, P < .05
ns - indicates not different from Control (untreated) group

Example 41: Suppression of Immune Responses to Therapeutic Nonhuman Antibodies by Chemical Modification

The therapeutic effectiveness of nonhuman antibodies is limited by the immune response that is potentially harmful to the patient. Serious complications may occur including serum sickness, anaphylactic symptoms, and deposition of toxic immune complexes in the liver (Abuchowski, A., "Effect of Covalently Attached Polyethylene Glycol on the Immunogenicity and Activity of Enzymes", Rutgers University, New Jersey, 1975; Sehon, A. H., "Suppression of Antibody Responses by Chemically Modified Antigens", <u>Int. Arch. Allergy Appl. Immunol.</u> 94 (1991):11-20). Two ways to obviate immunogenicity are to use human antibodies and to use genetically "humanized" animal antibodies in which CDRs, from a murine antibody for example, have been grafted onto a human antibody framework. Alternatively, antibodies can be chemically derivatized with nonimmunogenic, nonallergenic, nonantigenic molecules which mask the foreign protein and thereby suppress the host immune response (Abuchowski, A., "Effect of Covalently Attached Polyethylene Glycol on the Immunogenicity and Activity of Enzymes", Rutgers University, New Jersey, 1975; Sehon, A. H., "Suppression of Antibody Responses by Chemically Modified Antigens", <u>Int. Arch. Allergy Appl. Immunol.</u> 94 (1991):11-20). The host immune response can be substantially reduced by conjugation of foreign proteins to, for example, copolymers of D-

glutamic acid and D-lysine (D-GL), polyethylene glycols (PEG), monomethoxypolyethylene glycols (mPEG), or polyvinyl alcohols (PVA) (Sehon, A. H., "Suppression of the IgE Antibody Responses with Tolerogenic Conjugates of Allergens and Haptens", In Progress In Allergy, Vol. 32 (1982):161-202). In each case, a protein such as an antibody (Ab) is modified with multiple molecules (n) of the conjugate; i.e. $Ab(PEG)_n$. The suppression of the immune response depends on an optimum value of n; if n is too small or too large the effect is not as substantial (Jackson, C. and J. C., Charlton, J. L., Kuzminski, K., Lang, G. M., Sehon, A. H., "Synthesis, Isolation, and Characterization of Conjugates of Ovalbumin with Monomethoxypolyethylene Glycol using Cyanuric Chloride as the Coupling Agent", Anal. Biochem. 165 (1987):114-127). The optimal value of n can be determined without undue experimentation by one skilled in the art by preparing antibodies with different values of n and determining the immunogenicity of each modified antibody in a host animal.

Conjugation of a catalytic antibody or catalytic/tumor-binding bispecific antibody to nonantigenic molecules can be carried out as follows (Jackson, C. and J. C., Charlton, J. L., Kuzminski, K., Lang, G. M., Sehon, A. H., "Synthesis, Isolation, and Characterization of Conjugates of Ovalbumin with Monomethoxypolyethylene Glycol using Cyanuric Chloride as the Coupling Agent", Anal. Biochem. 165 (1987):114-127). The optimum value of n (see above) is determined experimentally by one skilled in the art and the procedure can be varied to achieve this degree of conjugation. Preferably the antibody is conjugated to mPEG, although other conjugates may also provide the desired effect. mPEG is preferred over PEG because PEG has two terminal hydroxyl groups which may participate in undesirable intra- and inter-molecular crosslinking of conjugates (Sehon, A. H., "Suppression of Antibody Responses by Chemically Modified Antigens", Int. Arch. Allergy Appl. Immunol. 94 (1991):11-20). The type of mPEG, for example $mPEG_6$ (average molecular weight = 6000) or $mPEG_{20}$ (average molecular weight = 20,000) may also be chosen without undue experimentation. Additionally, the scale of the procedure is altered accordingly, depending on how much conjugated antibody is available or required.

Preparation of the mPEG-conjugated antibody consists of two main steps;

1. Preparation of an active intermediate, 2-O-mPEG-4,6-dichloro-s-triazine ("mPEG intermediate").
2. The mPEG intermediate is reacted in the correct proportions with the antibody to form a conjugate with the desired value of n.

Because cyanuric chloride and the mPEG intermediate are exceedingly susceptible to hydrolysis, all reagents must be completely anhydrous and protected from atmospheric moisture. mPEG (20 g) is dissolved in anhydrous benzene (320 mL) at 80° C. Any moisture that may be associated with mPEG is removed by distillation of the benzene to approximately 160 mL. Under a nitrogen atmosphere, excess cyanuric chloride (6.64 g, recrystallized from anhydrous benzene), is added, followed by potassium carbonate (4.0 g, anhydrous, powdered) and the mixture is stirred at room temperature for 15 hours. Following this, the mixture is filtered through a sintered glass filter (under nitrogen). The filtrate is mixed with anhydrous petroleum ether (200 mL) to precipitate the mPEG intermediate, which is separated from reactants by filtration through a sintered glass filter under nitrogen. The precipitate is dissolved in 150 mL benzene and again precipitated with petroleum ether. This is repeated seven times to remove all residual cyanuric chloride. The mPEG intermediate is then dissoved in benzene, frozen at -78° C. The benzene is sublimated under high vacuum, to leave a white powder (mPEG intermediate). The mPEG intermediate can be stored in nitrogen in sealed vials (1 g or less per vial) at -60° C.

To obtain the antibody-mPEG conjugate (Ab(mPEG)) of varying n, different amounts of mPEG intermediate is added to 40 mg Ab, which is dissolved in sodium tetraborate (4 mL, 0.1 M, pH 9.2). The mixture is stirred for 30 minutes at 4° C, then for 30 minutes at room temperature.

Following conjugation, the mixture is passed through a Sephadex G-25 column (2.5 x 40 cm) equilibrated with 25 mM Tris buffer, pH 8.0. The conjugates are finally purified on a DEAE-Trisacryl column (5 x 40 cm) pre-equilibrated in 25 mM Tris, pH 8.0. The protein is bound to the column in this starting buffer, followed by a wash in the same Tris buffer. The proteins are eluted with a linear salt gradient ending in 50 mM NaCl, 25 mM Tris, pH 8.0.

Example 42: Production And Application Of A Bispecific Antibody That Targets A Tumor Antigen And Activates A Prodrug Into An Active Anticancer Drug At The Tumor

The prodrug 5'-O-(2,6-dimethoxybenzoyl-5-fluorouridine), compound **1c** in example 1a was prepared as described in Example 1a and tested for toxicity in mice. The toxicity *in vivo* of the prodrug as measured by effect on segmented neutrophils counts was substantially better than 50 times less toxic than the drug 5-Fluorouridine. The transition state analogue, the phosphonate ester of dimethoxy benzoyl fluorouridine compound **155** is prepared as described in Example 44. After conjugation of the phosphonate analogue to the carrier protein, keyhole limpet hemocyanin it is used to immunize mice and to produce monoclonal antibodies using traditional procedures. In addition, the spleens from mice with high titre antisera are used as a source of polyadenylated RNA. The RNA is primed with oligonucleotide primers complementary to mouse immunoglobulin families in a PCR amplification protocol. The PCR products are cloned into the *fd* phage vectors as described in Patent Application WO 92/01047 incorporated herein by reference. The resulting phage library and monoclonal antibodies produced in the traditional fashion are screened for binding to the transition state analog using procedures described in the literature. Candidate antibodies with the potential of being catalytic are screened for catalysis as described in the section above titled "Screening Antibodies for Esterase Catalytic Activity".

Bispecific single chain antibodies are produced using the following methods. A monoclonal specific for the cancer of interest, in this instance, B72.3, or other tumor specific antibody well known in the art, is cloned using methods already described. The antibody is cloned into the form of a single chain and characterized by expression in vectors known in the art. This single chain antibody gene is then combined with a single chain gene for the catalytic antibody isolated as described above. The linking of these two single chain genes is in the form of the linkers already described for the combination of the single chains or other sequences known to be involved with the linkage of antibody domains; specifically genes coding for $(ser\text{-}lys\text{-}ser\text{-}thr\text{-}ser)_3$, or hinge regions. These linked genes are then placed into an expression vector; in this instance, the vector pRC/CMS from In Vitrogen Inc., or other similar expression vectors known in the art. The introduction of this bispecific single chain gene into the expression vector is followed by the introduction of the combined vector into the host for that expression vector, in the case of this pRC/CMS vector, mammalian cells are the host. It will be appreciated that many host vector systems exist and have certain merits well known in the art. As examples of these systems, *E. coli*, yeast, and insect cells are extensions well known in the art of the system described above.

The recovery of expressed bispecific single chain is performed by protein purification methods known in the art, the recovered protein is characterized by the determination of the specific activity of the catalytic activity and the binding activity in combination with the catalytic activity to determine the purity of the material for treatment of tumors both in animals and man. The antibodies elicited by traditional monoclonal methods and by the phage library technique both bind to and cleave compound **1c**, as do the purified bispecific single chain (bivalent) antibody.

The bivalent antibody and the prodrug are formulated and administered as described above in the section titled "Formulation and Administration".

Example 43: Preparation of the Hapten of the Prodrug 141 in Example 38, the Linear Phosphonate of 5'-0-(2,4,6-Trimethoxybenzoyl)-5-fluorouridine, Compound 152

Refer to Figure 49 for the bold numbered compounds in this Example.

1,3,5-Trimethoxybenzene is lithiated with *n*-butyllithium and then reacted with N,N-diisopropylmethyl phosphonamidic chloride to give **150**. Subsequent condensation with 3*f* in the presence of tetrazole and *in situ* oxidation with *m*CPBA affords **151**. Removal of all protecting groups using thiophenol, catalytic hydrogenation and ammonium hydroxide give the prodrug hapten **152**.

<u>In detail</u>, the synthesis is as follows:

<u>Compound **150**</u>

n-Butyllithium (2-5 M is hexane, 1 mmol) is added to a solution of 1,3,5-trimethoxybenzene (1 mmol) in 2 mL of THF, while maintaining the temperature of the mixture below 0° C. After the addition is completed, the mixture is stirred at 0° C for a further 2 hours. It is then cooled to -78° C whereupon N,N-Diisopropylmethyl phosphonamidic chloride (1 mmol) is added. After the addition is completed, the mixture is stirred at -78° C for a further 2 hours. Triethylamine (5 ml) in EtOAc (45 ml) is added and the mixture is poured into saturated sodium bicarbonate (75 ml). The organic phase was further worked with saturated sodium bicarbonate (75 ml), brine (50 ml), dried over anhydrous Na_2SO_4, concentrated *in vacuo*, redissolved in triethylamine (0.3 ml) in hexane (2.7 ml), and purified by flash chromatography using 10% triethylamine in hexane to give the product (compound **150**) as a colorless solid.

Compound **151**

Compound **150** (1 mmol) is dissolved in 3 mL of CH_2Cl_2 and compound 3*f* (0.20 mmol) followed by tetrazole (2.5 mmol) are added. After one hour, *m*CPBA (1.25 mmol) is added and the mixture is stirred for a further 15 minutes, poured into saturated NH_4Cl (30 mL) and extracted with EtOAc (2 x 50 mL). Organic phases are dried over anhydrous $MgSO_4$, and concentrated *in vacuo*. The mixture is purified by flash chromatography to give the product, compound **151**.

Compound **152**

Compound **151** (1 mmol) is dissolved in 1 mL of dioxan and a solution of thiophenol (10 mmol) and triethylamine (10 mmol) in dioxan (5 mL) is added. The mixture is stirred for 16 hours. It is then concentrated *in vacuo*, redissolved in 2 mL of CH_2Cl_2 and added dropwise to 300 mL of petroleum ether with stirring. The precipitate is collected after decanting and is redissolved in 2 mL of CH_2Cl_2 and again added dropwise to another 300 mL of petroleum ether with stirring. The precipitate is again collected after decanting, redissolved in 20 mL of EtOAc, 5% Pd-C (10 weight %) is added and the mixture is stirred at room temperature under an atmosphere of hydrogen until uptake of hydrogen is complete. The catalyst is removed by filtration through a pad of celite, washing with methanol. The filtrate is collected, concentrated *in vacuo* and a solution of this hydrogenated compound (1 mmol) and ammonium hydroxide (10 mL) in methanol (10 mL) is heated in a sealed tube for overnight. After completion of the reaction solvents are removed *in vacuo* and the product is purified by reverse phase HPLC to give compound **152**.

Example 44: Preparation of the Hapten for the Prodrug 1c in Example 1a, the Linear Phosphonate of 5'-0-(2,6-dimethoxybenzoyl)-5-fluorouridine, Compound 155

Refer to Figure 50 for the bold numbered compounds in this Example.

1,5-Dimethoxybenzene is lithiated with *n*-butyllithium and then reacted with N,N-diisopropylmethyl phosphonamidic chloride to give **150**. Subsequent condensation with 3*f* in the presence of tetrazole and *in situ* oxidation with *m*CPBA affords **151**. Removal of all protecting groups using thiophenol, catalytic hydrogenation, and ammonium hydroxide gives the prodrug hapten **152**.

In detail, the synthesis is as follows:

Compound 153

n-Butyllithium (2-5 M is hexane, 1 mmol) is added to a solution of 1,5-dimethoxybenzene (1 mmol) in 2 mL of THF, while maintaining the temperature of the mixture below 0° C. After the addition is completed, the mixture is stirred at 0° C for a further 2 hours. It is then cooled to -78° C whereupon N,N-Diisopropylmethyl phosphonamidic chloride (1 mmol) is added. After the addition is completed, the mixture is stirred at -78° C for a further 2 hours. Triethylamine (5 ml) in EtOAc (45 ml) is added and the mixture is poured into saturated sodium bicarbonate (75 ml). The organic phase was further worked with saturated sodium bicarbonate (75 ml), brine (50 ml), dried over anhydrous Na_2SO_4, concentrated *in vacuo*, redissolved in triethylamine (0.3 ml) in hexane (2.7 ml), and purified by flash chromatography using 10% triethylamine in hexane to give the product (compound **153**) as a colorless solid.

Compound 154

Compound **153** (1 mmol) is dissolved in 3 mL of CH_2Cl_2 and compound 3*f* (0.20 mmol) followed by tetrazole (2.5 mmol) are added. After one hour, *m*CPBA (1.25 mmol) is added and the mixture is stirred for a further 15 minutes, poured into saturated NH_4Cl (30 mL) and extracted with EtOAc (2 x 50 mL). Organic phases are dried over anhydrous $MgSO_4$, and

concentrated *in vacuo*. The mixture is purified by flash chromatography to give the product, compound **154**.

Compound 155

Compound **154** (1 mmol) is dissolved in 1 mL of dioxan and a solution of thiophenol (10 mmol) and triethylamine (10 mmol) in dioxan (5 mL) is added. The mixture is stirred for 16 hours. It is then concentrated *in vacuo*, redissolved in 2 mL of CH_2Cl_2 and added dropwise to 300 mL of petroleum ether with stirring. The precipitate is collected after decanting and is redissolved in 2 mL of CH_2Cl_2 and again added dropwise to another 300 mL of petroleum ether with stirring. The precipitate is again collected after decanting, redissolved in 20 mL of EtOAc, 5% Pd-C (10 weight %) is added and the mixture is stirred at room temperature under an atmosphere of hydrogen until uptake of hydrogen is complete. The catalyst is removed by filtration through a pad of celite, washing with methanol. The filtrate is collected, concentrated *in vacuo* and a solution of this hydrogenated compound (1 mmol) and ammonium hydroxide (10 mL) in methanol (10 mL) is heated in a sealed tube for overnight. After completion of the reaction solvents are removed *in vacuo* and the product is purified by reverse phase HPLC to give compound **155**.

*   *   *

The foregoing is intended as illustrative of the present invention but not limiting. Numerous variations and modifications may be effected without departing from the true spirit and scope of the invention.